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The bi-functional nature of plant 5,10 methylenetetrahydrofolate dehydrogenase-5,10-methenyltetrahydrofolate cyclohydrolase and the characterization of 10-formyltetrahydrofolate synthetase in photosynthetic tissues

by

Liangfu Chen ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of *Doctor of Philosophy.*

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "The bi-functional nature of plant 5,10-methylenetetrahydrofolate dehydrogenase-5,10-methenyltetrahydrofolate cyclohydrolase and the characterization of 10-formyltetrahydrofolate synthetase in photosynthetic tissues" submitted by Liangfu Chen in partial fulfillment of the requirements for the degree of *Doctor of Philosophy in Physiology and Cell Biology*.

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To my family

Abstract

Folate-dependent one-carbon metabolism has importance in the formation of purines, thymidylate, serine, methionine and formylmethionyl-tRNA. In yeast and mammalian cells, the formation of 10-formyltetrahydrofolate and its conversion to 5,10-methylenetetrahydrofolate are catalyzed by C_1-THF synthase, a tri-functional protein possessing 10-formyltetrahydrofolate synthetase (SYN), 5,10-methenyltetrahydrofolate cyclohydrolase (CYC), and 5,10-methylenetetrahydrofolate dehydrogenase (DHY) activities. The structural organization and subcellular distribution of these activities in higher plants were investigated in the present studies.

In leaf extracts of 14 day pea *(Pisum sativum* L. cv. Homesteader) seedlings, these three activities were mainly associated with the cytosolic fraction with less than 1% of each activity being associated with mitochondria purified on a Percoll gradient. Fractionation of whole leaf homogenates resulted in the co-purification of DHY and CYC (subunit M_r = 38,000) and the isolation of a SYN protein (subunit M_r = 66,000). Polyclonal antibodies were raised against purified cytosolic DHY-CYC (DHY-CYC-Ab) and cytosolic SYN (SYN-Ab) respectively. Immunoblots showed that DHY-CYC-Ab cross-reacted with a mitochondrial protein band of M_r = 38,000. In mitochondrial extracts, two protein bands (subunit M_r = 40,000 and 44,000) cross-reacted with SYN-Ab. Immunoaffinity chromatography (DHY-CYC-Ab as the immobile ligand) indicated that the bulk of mitochondrial SYN activity was not associated with mitochondrial DHY or CYC.

When 9-d etiolated pea seedlings were exposed to light for up to 3 days, the specific enzyme activities of DHY-CYC, in whole-leaf extracts, rose two-fold and more DHY-CYC-Ab cross-reacting protein was detected. In contrast, the specific activity of SYN fell from 5 to 1 μmol min^{-1} mg^{-1} protein and less SYN-Ab cross-reacting protein was detected. When extracts of other higher plants were examined by column chromatography and immunological studies, it appeared that the bi-functional DHY-CYC and mono-functional SYN might have widespread occurrence.

A pea leaf λgt11 cDNA expression library was screened using DHY-CYC-Ab. The open reading frame of the isolated cDNA (1219 bp) encoded a protein of 294 amino acids. The deduced primary structure of this plant DHY-CYC showed most of the homologous regions found in the DHY-CYC enzymes or domains of seven other biological sources. The data suggest that in higher plants these folate-dependent enzymes have a structural organization that is distinct from that reported for other eukaryotic species.

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List of Abbreviations

A	absorbance
ADP	adenosine diphosphate
AICAR	5-phosphoribosyl-5-amino-4-imidazole carboxamide
Ampr	ampicilin resistance
ATP	adenosine triphosphate
BES	N,N-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid
bp; kb	base-pairs; kilo-base-pairs
BSA	bovine serum albumin
C_1-THF Synthase	trifunctional C_1 tetrahydrofolate synthase
cDNA	complementary DNA
CHO	chinese hamster ovary
Ci; µCi; mCi	Curie; microcurie; millicurie
CTAB	hexadecyltrimethylammonium bromide
Da; kDa	Daltons; kiloDaltons
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dITP	2'-deoxyinosine 5'-triphosphate
dNTP	2'-deoxynucleoside triphosphate

dTTP	2'-deoxythymidine 5'-triphosphate
DEAE	diethylaminoethyl
DEPC	diethyl pyrocarbonate
dH_2O; ddH_2O	distilled water; double distilled water
DHFR	dihydrofolate reductase
DHFS	dihydrofolate synthetase
DHPS	dihydropteroate synthase
DHY-CYC	5,10-methylenetetrahydrofolate dehydrogenase-5,10-methenyltetrahydro-folate cyclohydrolase bifunctional protein
DHY-CYC-Ab	polyclonal antibodies raised against the cytosolic form of DHY-CYC
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immuno-sorbent assay
fMet-tRNA	formyl-methionyl transfer RNA
FPGS	folylpolyglutamate synthetase
GAR	glycinamide ribonucleotide
GDC	glycine decarboxylase
GGH	gamma glutamyl hydrolase

HEPES	4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid
HPLC	high performance liquid chromatography
HPPK	6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase
H_2PteGlu	7,8-dihydropteroylglutamate; dihydrofolate
H_4PteGlu	5,6,7,8-tetrahydropteroylglutamate; tetrahydrofolate
H_4PteGlu$_n$	5,6,7,8-tetrahydropteroyl-poly-γ-glutamate, where n = the number of L-glutamate moieties
10-HCO-H_4PteGlu$_n$	10-formyltetrahydropteroylpolyglutamate
5,10-CH_2-H_4PteGlu$_n$	5,10-methylenetetrahydropteroyl-polyglutamate
5,10-CH^+-H_4PteGlu$_n$	5,10-methenyltetrahydropteroyl-polyglutamate
hr; min	hours; minutes
IPTG	isopropyl-β-D-thiogalactoside
MOPS	3-(N-morpholino) propanesulphonic acid
MTX	methotrexate
NAD	nicotinamide adenosine dinucleotide
NADP	nicotinamide adenosine dinucleotide phosphate
N. crassa	*Neurospora crassa*
p-ABA	p-aminobenzoate
p-ABAGlu$_n$	p-aminobenzoyl polyglutamate
PAGE	polyacrylamide get electrophoresis

PBS	phosphate buffered saline
PBST	phosphate buffered saline with 0.1% Tween 20
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming units
Pi	inorganic orthophosphate
PMSF	phenylmethylsulphonylfluoride
PteGlu	pteroylglutamic acid
RH	relative humidity
RNA	ribonucleic acid
RNase	ribonuclease
tRNA	transfer ribonucleic acid
SHMT	serine hydroxymethyltransferase
SDS	sodium dodecyl sulphate
SM	suspending medium for phage lysate
SYN	10-formyltetrahydrofolate synthetase
SYN-Ab	polyclonal antibodies raised to the cytosolic form of SYN
THFA	tetrahydrofolic acid
Tris	tris (hydroxymethyl) aminomethane
TS	thymidylate synthase

1. INTRODUCTION

One-carbon metabolism has importance in the formation of purines, thymidylate, serine, methionine and formylmethionyl-tRNA (Blakley, 1969; Rowe, 1984; Santi and Danenberg, 1984; MacKenzie, 1984; Matthews, 1984; Staben and Rabinowitz, 1984). In these syntheses, single-carbon groups, ranging in oxidation state from formyl to methyl, are donated by tetrahydrofolate polyglutamates ($H_4PteGlu_n$). Living cells contain a number of one-carbon substituted $H_4PteGlu_n$ derivatives (Cossins, 1984; Shane, 1989), which actively participate in folate-dependent pathways (Figure 1). Enzymes catalyzing these reactions have received detailed study (MacKenzie, 1984; Schirch, 1984; McGuire and Coward, 1984; Matthews, 1984; Staben and Rabinowitz, 1984). More recently, work in this field has intensified with the development of new and improved methods for folate isolation and characterization. In particular, important advances have been made by the cloning, sequencing and site-directed mutagenesis of genes that encode the major enzymes of folate metabolism.

The nutritional and clinical importance of folates has provided special impetus for work on mammalian cells. Several topics related to these fields have been reviewed. These include human folate deficiencies and the bioavailability of folate (Herbert, 1990; Gregory, 1997), folate receptors and folate-binding proteins (Kane and Waxman, 1989; Henderson, 1990; Antony, 1996), folate and antifolate transport (Horne, 1993), the interaction of enzymes with folylpolyglutamates (Schirch and Strong, 1989), the development of new antifolates (Fleming and Schilsky, 1992; Schultz, 1995; Chu et al.,

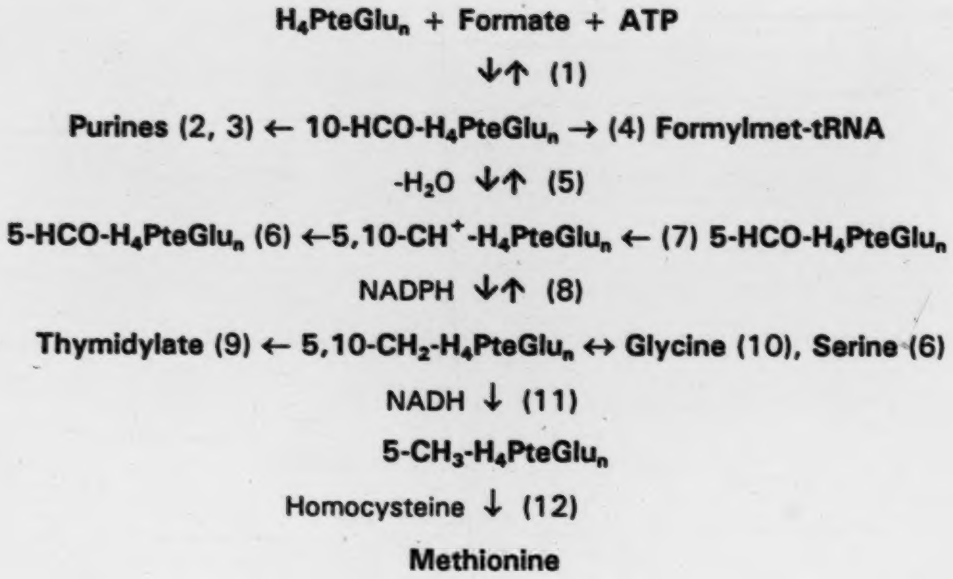

$H_4PteGlu_n$ + Formate + ATP
↓↑ (1)
Purines (2, 3) ← $10\text{-}HCO\text{-}H_4PteGlu_n$ → (4) Formylmet-tRNA
$-H_2O$ ↓↑ (5)
$5\text{-}HCO\text{-}H_4PteGlu_n$ (6) ← $5{,}10\text{-}CH^+\text{-}H_4PteGlu_n$ ← (7) $5\text{-}HCO\text{-}H_4PteGlu_n$
NADPH ↓↑ (8)
Thymidylate (9) ← $5{,}10\text{-}CH_2\text{-}H_4PteGlu_n$ ↔ Glycine (10), Serine (6)
NADH ↓ (11)
$5\text{-}CH_3\text{-}H_4PteGlu_n$
Homocysteine ↓ (12)
Methionine

Figure 1. *Major Folate-dependent Pathways of One-carbon Metabolism.*

Enzymes catalyzing individual reactions are: (1) 10-formyltetrahydrofolate synthetase (EC 6.3.4.3); (2) phosphoribosylglycinamide formyltransferase (EC 2.1.2.2); (3) phosphoribosylaminoimidazolecarboxamide formyltransferase(EC 2.1.2.3); (4) methionyl-tRNA formyltransferase (EC 2.1.2.9); (5) 5,10-methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9); (6), serine hydroxymethyltransferase (EC 2.1.2.1); (7) 5,10-methenyltetrahydrofolate synthetase (EC 6.3.3.2); (8) 5,10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5); (9), thymidylate synthase (EC 2.1.1.45) ; (10) glycine cleavage complex (EC 1.4.4.2/2.1.2.10); (11) 5,10-methylenetetrahydrofolate reductase (EC 1.5.1.20); and (12) methionine synthase (EC 2.1.1.14).

1996), folate status and carcinogenesis (Glynn and Albanes, 1994), the importance of folate, antifolates and folate analogs in oncology (Hum and Kamen, 1996), the metabolic role of leucovorin (5-HCO-H_4PteGlu) (Stover and Schirch, 1993), the biosynthesis and regulatory roles of folylpolyglutamates (Shane, 1989), and the compartmentation of one-carbon metabolism (Appling, 1991, Wagner, 1996).

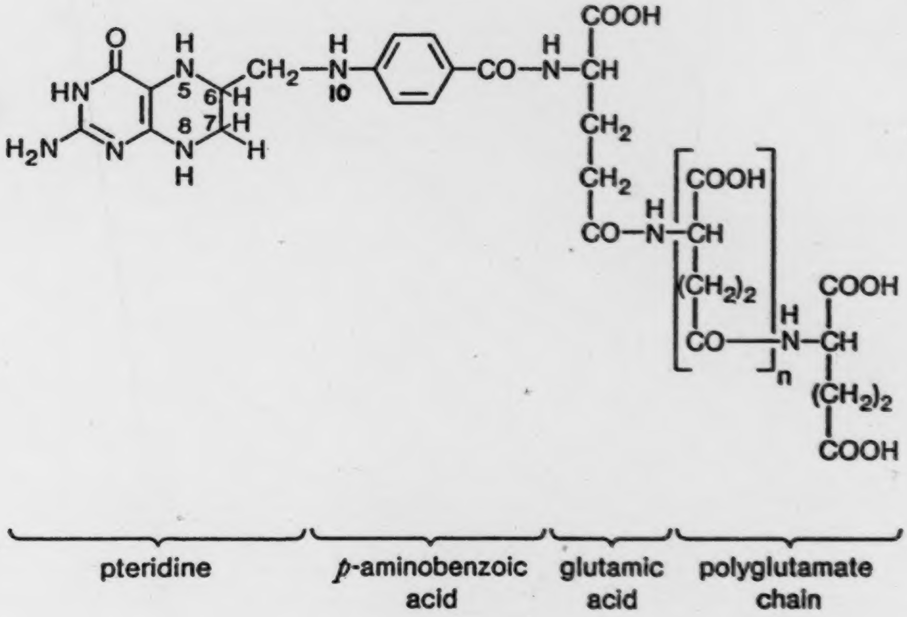
The present literature review focuses on folate biochemistry and one carbon metabolism with emphasis on the role of C_1-THF synthase. Where possible, details of investigations of higher plant systems are highlighted. For convenience, this Introduction is divided into three main sections. Firstly, the biochemistry of folates including the discovery of folic acid, the nature of folates and the biosynthesis of tetrahydrofolate are described. Secondly, the area of one-carbon metabolism is reviewed to include the generation, interconversion and utilization of C_1 units. This section also discusses the compartmentation of one-carbon metabolism in eukaryotic species. Thirdly, the role of C_1-THF synthase in the generation and interconversion of C_1 units is reviewed. This last section emphasizes studies of C_1-THF synthase and its associated activities in mammalian, yeast, and prokaryotic cells. The relatively limited knowledge of this topic in higher plants is also noted in this section.

1.1. The Biochemistry of Folates

1.1.1. Discovery of folic acid and the chemical nature of naturally occurring folates

A vitamin, later identified as pteroylglutamic acid (PteGlu), was first reported as an antianemia agent in animals and as a growth factor in bacteria (Blakley, 1969). The factor was first isolated from spinach leaves by Mitchell et al. (1941), who called it folic acid. These workers subsequently described the basic chemical and physiological properties of folic acid as well as methods for its purification from spinach leaves (Blakley, 1969). Folic acid stimulates the growth of *Lactobacillus casei* and *Streptococcus faecalis*, and these bacteria were used in the development of sensitive microbiological assay methods (Blakley, 1969). Many tissues were subsequently examined for this new vitamin and evidence accumulated that folate-related compounds are normal constituents of living cells (for review see Cossins, 1984). The chemical composition of this factor was described by Mowat et al. (1948).

As a class of closely related compounds, folates contain three major constituents, a pteridine ring; a p-aminobenzoate and at least one L-glutamate moiety (Figure 2). Studies of living organisms show that folates tend to exist predominantly in reduced forms (Blakley, 1969). Therefore the tetrahydrofolate form (H_4PteGlu), reduced at positions 5, 6, 7, and 8 of the pteridine ring (Figure 2), usually represents the bulk of cellular folates. This chemical form of folate is also an active coenzyme in various folate-dependent metabolic pathways. In one-carbon metabolism, C_1-units are acquired or substituted by the oxidation or reduction of carbon units at the N-5 and N-10 positions of the pteridine ring to give 5-formyl-, 5-methyl-, 10-formyl-, 5,10-methynel- and 5,10-methylene derivatives (Blakley, 1969; Cossins, 1984; MacKenzie, 1984). In many enzyme-catalyzed reactions the folate molecule does not necessarily remain enzyme bound, but acts rather as a co-substrate (Shane, 1989). Consequently the ability of folate to act as a donor of one



pteridine
p-aminobenzoic acid
glutamic acid
polyglutamate chain

Figure 2. *Structure of the $H_4PteGlu_n$ molecule.*

Cellular folates are C_1-substituted at the N-5 and N-10 positions to give 5-formyl-, 5-methyl-, 10-formyl-, 5,10-methenyl- and 5,10-methylene derivatives. The polyglutamate chain usually contains 5-8 γ-glutamyl residues.

carbon units depends on the regeneration of the one-carbon moiety by other related enzymes in the metabolic pathway or cycle. Folates in solution tend to be very unstable, being sensitive to oxygen, light and extremes of pH (Mullins and Dutch, 1992).

There is now accumulated evidence to indicate that cellular folates (Figure 2) exist primarily as tetrahydropteroylpolyglutamates ($H_4PteGlu_n$) (McGuire and Coward, 1984; Shane, 1989). In most species, the polyglutamate chain contains 5 to 8 γ-glutamyl residues (Cossins, 1984; Shane, 1989). Furthermore, most folate-dependent enzymes display increased affinities and decreased K_m values for folylpolyglutamates as compared to folylmonoglutamates (McGuire and Coward, 1984; Shane, 1989; Schirch and Strong, 1989). The polyglutamate chain also plays an important role in the cellular retention of folates and the channeling of substrates between multi-functional protein complexes that catalyze folate-dependent syntheses (Shane, 1989).

1.1.2. The biosynthesis of tetrahydrofolate

The three structural components of folate, namely pteridine, p-aminobenzoate, and L-glutamate (Figure 2) are incorporated into $H_4PteGlu_n$ by a complex pathway (Figure 3). Since animals lack steps of folate biosynthesis prior to dihydrofolate reductase (Figure 3), they require a dietary source of the vitamin (Herbert 1990; Gregory, 1997). In contrast, plants and most microorganisms are able to synthesize tetrahydrofolate *de novo* by a pathway that starts with GTP and requires the sequential operation of five major enzymes (Reactions 3 to 7, Figure 3). For review of this topic see Shiota (1984); Cossins and Chen (1997).

Guanosine 5′-triphosphate

↓ [1]

L-*threo*-neopterin 2′,3′-cyclic phosphate

↓

6-hydroxymethyl dihydroneopterin

↓ [2]

2-amino-4-hydroxy-6-hydroxymethyl dihydropteridine

ATP ↓ [3]

2-amino-4-hydroxy-6-pyrophosphoryl-methyl dihydropteridine

p-ABA ↓ [4]

Dihydropteroate (H_2Pte)

ATP, glutamate ↓ [5]

Dihydrofolate (H_2PteGlu)

NADPH ↓ [6]

Tetrahydrofolate (H_4PteGlu)

ATP, glutamate ↓ [7]

Tetrahydrofolate polyglutamate (H_4PteGlu$_n$)

Figure 3. *Principal Steps in the Pathway for H_4PteGlu$_n$ Biosynthesis.*

The dihydropteroate precursor, 2-amino-4-hydroxy-6-pyrophosphoryl-methyl dihydropteridine, condenses with p-aminobenzoate in a reaction catalyzed by dihydropteroate synthase [4], (DHPS, EC 2.5.1.15). Other key enzymes are 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase [3], (HPPK, EC 2.7.6.3); dihydrofolate synthase [5], (DHFS, EC 6.3.2.12); dihydrofolate reductase [6], (DHFR, EC 1.5.1.3); and folylpolyglutamate synthetase [7], (FPGS, EC 6.3.2.17).

The reaction of this pathway, catalyzed by DHPS (Reaction 4, Figure 3), is the target of antimicrobial sulfonamide drugs. Consequently, considerable effort has been focused on the molecular characterization of these bacterial proteins (Lopes et al., 1987; Dallas et al., 1992; Bognar et al., 1987). There is now strong evidence, from recent enzyme and molecular cloning studies, that the folate biosynthetic pathway of plants, like that of *E. coli*, involves the intermediary formation of dihydropteroate (for review see Cossins and Chen, 1997). In this regard, a bifunctional HPPK (Reaction 3, Figure 3)/DHPS protein that occurs exclusively in the mitochondria has been purified from pea leaves (Rebeille et al., 1997). Consistent with the intracellular localization of this complex, molecular cloning resulted in the isolation of a cDNA with a nucleotide sequence for mitochondrial targeting (Rebeille et al., 1997). In addition, a homodimeric bifunctional DHFR/TS complex (Reactions 6, Figure 3; Reaction 9, Figure 1) was purified to homogeneity from extracts of pea leaf mitochondria (Neuburger et al., 1996). These workers also noted a mitochondrial DHFS activity (Reaction 5, Figure 3) and suggested that these organelles are probably a major site for folate biosynthesis in plants (Rebeille et al., 1997; Neuburger et al., 1996).

The formation of H_4PteGlu from H_2PteGlu, catalyzed by DHFR, has received detailed study (Blakley, 1984) and represents a target of several antifolate drugs used in cancer chemotherapy. In animals, fungi and bacteria, DHFR is a monomer of relatively low molecular weight (about 20 kDa) and is therefore particularly amenable to structural studies. As a result, information on DHFR structure is rapidly accumulating and, in wealth of detail, probably surpasses that of most other enzymes in folate metabolism

(Blakley, 1984). The DHFR gene has been cloned and the effects of site-directed mutagenesis have been assessed in a number of laboratories (Blakley, 1984; Fling et al., 1988; Barclay et al., 1988; Baccanari et al., 1989; Daved et al., 1992; Blakley et al., 1993; Erciken at al., 1993).

In higher plants, the *dhfr-ts* genes that encode DHFR/TS complexes in *Arabidopsis thaliana* (Lazar et al., 1993), *Daucus carota* (Luo et al., 1993) and *Glycine max* (Wang et al., 1995) have been cloned and sequenced. The coding regions for DHFR and TS are located at the N- and carboxy termini respectively. Furthermore, the deduced amino acid sequences of the DHFR and TS domains show striking similarities to those reported for the corresponding mono-functional enzymes of other eukaryotes. It is noteworthy that the studies of *A. thaliana* (Lazar et al., 1993) and carrot (Luo et al., 1997) also provided evidence for two distinct genes, each capable of encoding a DHFR/TS complex. As plant cells require DNA precursors in nuclei, mitochondria and chloroplasts, it is conceivable that different DHFR/TS complexes may occur in these compartments, but information on this possibility is still lacking (Cossins and Chen, 1997).

With the exception of some bacteria (Blakley, 1984; Fleming and Schilsky, 1992), DHFR proteins are strongly inhibited by antifolates such as methotrexate (MTX). These antifolates, by blocking DHFR and other folate-dependent enzyme activities, typically result in $H_4PteGlu_n$ deficiencies. In mammalian cells, MTX cytotoxicity is enhanced by its glutamyl conjugation, a reaction catalyzed by FPGS (Fleming and Schilsky, 1992). In contrast, there have been few studies of $MTXGlu_n$ formation by plant cells (Wu et al.,

1994) or of the folate deficiencies (Crosti et al., 1993; Imeson et al., 1988) caused when such cells are exposed to MTX or other antifolates.

As noted earlier, most folate-dependent enzymes exhibit a preference for γ-glutamyl conjugated folate substrates (McGuire and Coward, 1984; Schirch and Strong, 1989) and these polyglutamates are the principal forms of folate in living cells (Cossins, 1984; Shane, 1989). The importance of these derivatives in one-carbon metabolism is also supported by studies of mutant cell lines. Thus lesions which affect the expression of FPGS (Reaction 7, Figure 3), result in auxotrophies for products of one-carbon metabolism (for reviews see Shane, 1989; Cossins and Chen, 1997). Recent studies by Shane's group (Sussman et al., 1986; Garrow et al., 1992; Lowe et al., 1993; Lin et al., 1993; Kim et al, 1993; Chen et al., 1996) have centered on FPGS-deficient mammalian cell lines. A combination of molecular and enzyme studies has provided new insights into FPGS expression and the roles of mitochondrial folate metabolism. In this regard, these workers used an FPGS-deficient cell line (AUX B1) and transformations with either the human or *E. coli* FPGS genes to demonstrate that eukaryotic cells normally express cytosolic and mitochondrial FPGS proteins that are encoded by the same nuclear gene.

In some bacteria, the DHFS and FPGS reactions are catalyzed by a bifunctional protein encoded by the *folC* gene but in mammals, fungi, and plants, polyglutamate biosynthesis is catalyzed by mono-functional, monomeric proteins (M_r = ca 60,000) that have several properties in common (Cossins and Chen, 1997). These include alkaline pH optima, fairly broad specificity for folate substrates, and a dependency on Mg-ATP. A number of laboratories have now cloned and sequenced the FPGS gene. For example, the

E. coli folC gene that encodes DHFS/FPGS was first cloned by Bognar et al. (1985) and used to obtain the highly purified protein. Cloning of the *L. casei* FPGS gene has also allowed extensive work on the purification, crystallization and characterization of FPGS protein (Toy and Bognar, 1990; Cody et al., 1992). Plant cells appear to express cytosolic (Chan et al., 1986) and mitochondrial (Neuburger et al., 1996) FPGS activities but their relative contributions to folate biosynthesis and one-carbon metabolism have not been elucidated.

1.2. Pathways of One-carbon Metabolism

1.2.1. Folate-dependent serine metabolism (SHMT)

$$\text{Serine} + H_4\text{PteGlu}_n = 5,10\text{-}CH_2\text{-}H_4\text{PteGlu}_n + \text{glycine}$$

The serine hydroxymethyltransferase (SHMT) reaction results in the interconversion of serine and glycine (Reaction 6, Figure 1) and is a major source of 5,10-CH_2-H_4PteGlu$_n$ needed in the synthesis of purines, thymidylate, methionine and choline (Schirch, 1984). The enzyme has been purified and characterized from several prokaryotic and eukaryotic sources including plants (Schirch, 1984; Cossins, 1987). SHMT activities are present in the cytosolic and mitochondrial compartments of eukaryotes (Schirch, 1984; Appling, 1991).

There have now been numerous studies of bacterial and mammalian SHMT proteins. For bacteria, these investigations have included cloning of the SHMT (*glyA*) gene in *Salmonella typhimurum* (Urbanowski et al., 1984), and detailed characterization of *E. coli* SHMT (Schirch et al., 1985; Hopkins and Schirch, 1986; Schirch et al., 1993). Mammalian SHMT isoenzymes have also been characterized in terms of catalytic properties, primary structure and sequencing of the encoding nuclear genes (Schirch et al., 1986; Martini et al., 1987; Martini et al., 1989; Strong et al., 1989; Strong and Schirch, 1989; Strong et al., 1990; Stover and Schirch, 1990, 1991 and 1992; Schirch et al., 1991; Garrow et al., 1993; Stover et al., 1997). The SHMT proteins in *N. crassa* (Kruschwitz et al., 1993 and 1994; McClung et al., 1992; Jeong and Schirch, 1996) and yeast (McNeil et al., 1994 and 1996) have also been extensively studied.

Work on plant SHMT has concentrated on the mitochondrial isoenzyme of photorespiring leaves (Bourguignon et al., 1988; Vijaya et al., 1991; Turner et al., 1992 and 1993; Besson et al., 1993; Kopriva, 1995). In this regard, a mitochondrial SHMT protein (tetrameric, subunit $M_r = 53,000$) has been isolated from pea leaves and purified to apparent homogeneity (Turner et al., 1993). This matrix protein is clearly associated with glycine decarboxylase (GDC, Reaction 10, Figure 1) during photorespiration so that the production and utilization of 5,10-CH_2-H_4PteGlu reaches a steady state equilibrium. In such leaf mitochondria, it is conceivable that GDC and SHMT activities are closely linked via a soluble pool of 5,10-CH_2-H_4PteGlu$_n$. Consistent with this association, both enzymes display a strong preference for folylpolyglutamates (Besson et al., 1993).

There is also evidence that the SHMT of pea leaf mitochondria occurs as two forms that are immunologically related (Turner et al., 1992). This study also revealed a third, non-mitochondrial form of SHMT in leaf extracts. The deduced amino acid sequence of pea mitochondrial SHMT is similar to those of the cytosolic and mitochondrial SHMT isoenzymes of rabbit (Martini et al., 1987 and 1991). These homologies are also shown by the mitochondrial SHMT of *Solanum tuberosum* (Kopriva et al., 1995). Transcription of the mRNA encoding pea leaf mitochondrial SHMT is strongly induced by light and mature plants, placed in the dark, show lower levels of this mRNA species (Turner et al., 1993). Clearly, light controls SHMT expression in leaves but the existence of other regulatory mechanisms is still uncertain.

1.2.2. The glycine cleavage system (GDC)

$$\text{Glycine} + \text{NAD} + H_4\text{PteGlu}_n = 5,10\text{-}CH_2\text{-}H_4\text{PteGlu}_n + \text{NADH} + CO_2 + NH_3$$

The GDC reaction represents a major source of $5,10\text{-}CH_2\text{-}H_4\text{PteGlu}_n$ in most prokaryotic and eukaryotic species (Schirch, 1984). In animals, fungi and higher plants, the reaction is catalyzed by a complex of mitochondrial matrix proteins (Schirch, 1984; Douce and Neuburger, 1989; Douce et al., 1994). These include a dimeric, pyridoxal phosphate-binding protein (P-protein, subunit $M_r = 94,000$), a lipoic acid-containing protein (H-protein, $M_r = 15,000$), a tetrahydrofolate-binding protein (T-protein, $M_r = 41,000$) and a lipoamide dehydrogenase protein (L-protein, dimeric, subunit $M_r = 60,000$). The importance of this reaction in photorespiration has stimulated work on the structure, function and biogenesis of GDC in higher plant mitochondria (Douce and Neuburger, 1989; Douce et al., 1994; Oliver et al., 1990; Oliver and Raman, 1995) where the complex contains one L-protein dimer, two P-protein dimers, twenty-seven H-protein units and nine T-protein units (Oliver et al., 1990).

In yeast and mammalian cells, the GDC reaction is reversible (Appling, 1991; Schirch, 1984). In contrast, the GDC of pea leaf mitochondria favors glycine oxidation and a relatively large pool of 5,10-CH_2-H_4PteGlu$_n$ is maintained in the matrix space (Bourguignon et al., 1988). This polyglutamate derivative, which may account for 65-80% of the folate pool, is thought to drive the serine hydroxymethyltransferase (SHMT) reaction in the direction of serine synthesis (Rebeille et al., 1994). Although both enzymes have more affinity for polyglutamate than monoglutamate substrates (Besson et al., 1993), there is no direct evidence to suggest that channeling of 5,10-CH_2-H_4PteGlu$_n$ occurs between these closely related reactions (Bourguignon et al., 1988). Earlier studies (Cossins, 1980) suggested that 2 moles of glycine are consumed for each mole of serine produced during photorespiration. However, it is still not clear if part of the matrix pool of 5,10-CH_2-H_4PteGlu$_n$, formed during photorespiration, is converted to other folates such as 10-HCO-, 5-HCO- or 5-CH_3-H_4PteGlu$_n$.

The individual genes encoding the GDC subunit proteins have recently been cloned in several laboratories. For example, Oliver's group (Kim and Oliver, 1990) cloned and sequenced the cDNA encoding the H-protein in peas. Rawsthorne and coworkers (Turner et al., 1992) used a pea leaf cDNA library to isolate, clone and characterize the P-protein. Bourguignon et al. (1992) isolated and characterized a cDNA for the complete L-protein precursor. These workers also showed that the L-protein was a functional component of several other mitochondrial enzyme complexes. More recently, cloning and characterization of the T-protein have been accomplished by screening a λgt11 pea leaf cDNA library (Bourguignon et al., 1993). In other related work, the organization and expression of the genes encoding pea mitochondrial GDC have been examined (Turner et al.,1993). The mRNA species encoding SHMT and the subunits of GDC were found to be strongly induced when etiolated pea plants were placed in the light to undergo greening.

1.2.3. Purine ring biosynthesis (GAR, AICAR)

The *de novo* synthesis of purines requires 10-HCO-$H_4PteGlu_n$ for the formylation of purine ring precursors (Rowe, 1984; Henikoff, 1987; Zalkin and Dixon, 1992). These folate-dependent reactions are catalyzed by glycinamide ribonucleotide (GAR) transformylase (Reaction 2, Figure 1) and 5-phosphoribosyl-5-amino-4-imidazole carboxamide (AICAR) transformylase (Reaction 3, Figure 1) respectively.

5-Ribosylglycinamide + 10-HCO-$H_4PteGlu_n$ = 5-Phosphoribosyl-N-formylglycinamide + $H_4PteGlu_n$

5-phosphoribosyl-5-amino-4-imidazole carboxamide + 10-HCO-$H_4PteGlu_n$ = 5-Phosphoribosyl-5-formamido-4-imidazole carboxamide + $H_4PteGlu_n$

In *E. coli* and *B. subtilis,* two distinct GAR transformylases, 10-HCO-$H_4PteGlu_n$- and formate-dependent, are expressed during *de novo* purine biosynthesis (for review see Cossins and Chen, 1997). A 10-formyltetrahydrofolate hydrolase appears to generate formate for this latter reaction (Nagy et al., 1993). In eukaryotes, 10-HCO-$H_4PteGlu_n$-dependent GAR transformylase activity is commonly associated with other enzymes of *de novo* purine biosynthesis (Henikoff, 1987; Zalkin and Dixon, 1992). There are however exceptions reported in yeast (White et al., 1985) and the higher plant *Arabidopsis*

(Schnoor, 1994), where cDNA encoding a mono-functional GAR transformylase have been isolated. Like GAR transformylase, AICAR transformylase activity is also associated with other enzymes of purine biosynthesis (Mueller and Benkovic, 1981) or one-carbon metabolism (Smith et al., 1981). To date there have been relatively few reports of AICAR transformylase in higher plants. This enzyme occurs in mitochondrial and plastid extracts of cowpea nodules (Atkins, et al., 1997). A detailed characterization of this plant protein has not been reported to date.

1.2.4. Methionine biosynthesis

In plants, the terminal reaction of methionine synthesis involves a folylpolyglutamate-dependent transmethylation of L-homocycsteine (Reaction 12, Figure 1). This

$$5\text{-CH}_3\text{-H}_4\text{PteGlu}_n + \text{L-homocycteine} \rightarrow \text{L-methionine} + \text{H}_4\text{PteGlu}_n$$

reaction is catalyzed by a cobalamin-independent methionine synthase (for review see Cossins, 1987). In *Neurospora*, this reaction appears to require $5\text{-CH}_3\text{-H}_4\text{PteGlu}_n$ as genetic lesions that result in folylpolyglutamate deficiencies cause an inability to form methionine (Cossins and Chan, 1984; Chan and Cossins, 1984). In contrast, the corresponding enzymes of mammalian cells and some bacteria are cobalamin-dependent (Matthews, 1984). On the other hand, *E. coli* expresses cobalamin-dependent and independent methionine synthases (Banerjee and Matthews, 1990; Banerjee et al., 1990).

To date, plant methionine synthases have received little study but those of bacteria and various eukaryotic species have been the subject of much investigation. In all species, the folate substrate for this transmethylation is generated by 5,10-CH_2-H_4PteGlu reductase (Reaction 11, Figure 1; Matthews, 1984). This reaction is physiologically irreversible and

$$5,10\text{-}CH_2\text{-}H_4PteGlu_n + FADH_2 \rightarrow 5\text{-}CH_3\text{-}H_4PteGlu_n + FAD$$

represents the only route for the *de novo* formation of the methyl group of methionine. Consequently, C_1- units are essentially committed to methioinine synthesis, as 5-CH_3-H_4PteGlu$_n$, the major circulatory form of folate, has no other known metabolic fate (Rozen, 1996). Recent clinical work suggested that deficiencies in 5,10-CH_2-H_4PteGlu$_n$ reductase are a major genetic abnormality in homocycteine metabolism (Rozen, 1996).

1.2.5. Thymidylate biosynthesis (TS)

Folate derivatives have importance in nucleic acid biosynthesis through a direct involvement in thymidylate production (Santi and Danenberg, 1984). Thymidylate synthase catalyzes the conversion of deoxyuridine monophosphate and 5,10-CH_2-H_4PteGlu$_n$ to thymidylate and H_2PteGlu$_n$. In this reaction, 5,10-CH_2-H_4PteGlu$_n$ serves as both the one-carbon donor and reductant, the 6-hydrogen of the cofactor being quantitatively transferred to the methyl group of thymidylate (Santi and Danenberg, 1984). This enzyme is unique in that it represents the sole *de novo* path for thymidylate synthesis and is the only enzyme that uses a folate cofactor in which the H_4PteGlu$_n$ is oxidized during one-carbon transfer (Santi and Danenberg, 1984). For a sustained synthesis of the pyrimidine product, it is therefore necessary to regenerate the reduced form of folate.

This is achieved by DHFR, one of the key enzymes in $H_4PteGlu_n$ biosynthesis (Blakley, 1984).

In bacteriophages, bacteria, yeast, and vertebrates, TS and DHFR occur as separate mono-functional enzymes (Santi and Danenberg, 1984; Appling, 1991). Some higher plants and protozoans however, as described earlier, express a bi-functional DHFR/TS protein. As DHFR has a major role in the reduction of $H_2PteGlu_n$ that arises during thymidylate biosynthesis (Blakley, 1984), it follows that DHFR/TS complex probably facilitates channeling of folate needed for the generation of 5,10-CH_2-$H_4PteGlu_n$. Kinetic analysis of the protozoan enzyme revealed very efficient channeling of the $H_2PteGlu$ produced by TS to the DHFR domain (Meek et al., 1985). The channeling of polyglutamate derivatives was however not reported by these workers.

1.2.6. The initiation of protein synthesis in bacteria, mitochondria and chloroplasts

In bacteria, polypeptide synthesis is initiated by formylmethionyl-tRNA (Kozak, 1983; Staben and Rabinowitz, 1984). In eukaryotes this initiator is required for protein synthesis in mitochondria and chloroplasts (Staben and Rabinowitz, 1984; Coffin and Cossins, 1986). Protein synthesis and C_1 metabolism are closely linked as the initiator receives formyl groups exclusively from 10-HCO-$H_4PteGlu$. The folate-dependent generation of formylmethionyl-tRNA is catalyzed by methionyl-tRNA formyltransferase

$$\text{10-HCO-}H_4\text{PteGlu} + \text{Methionyl-tRNA} = \text{Formylmethionyl-tRNA} + H_4\text{PteGlu}$$

(Reaction 4, Figure 1). The enzyme has been isolated and characterized from a variety of organisms, including plant species (for review see Cossins, 1987).

1.2.7. The compartmentation of folate-dependent pathways in eukaroytes

The folate-mediated one-carbon metabolism of eukaryotic cells is highly compartmentalized (Figure 4; Appling, 1991; Wagner, 1996). In this regard, there is evidence for organellar and cytosolic pools of folates (Wagner, 1996) as well as an intracellular distribution of folate-dependent enzymes (Appling, 1991, Wagner 1996). These authors have presented evidence for three types of compartmentation involved in the folate-mediated one-carbon metabolism of eukaryotic cells. Firstly, intracelluar compartmentation based on the cytoplasm and mitochondria (Figure 4). Secondly, a distribution of folate metabolites between free and protein-bound states. Thirdly, substrate channeling whereby metabolic intermediates undergo flux through sequential enzyme reactions. The realization that folates are highly compartmentalized implies that the folate-mediated one-carbon metabolism of eukaryotic cells is highly integrated and dynamic (Appling, 1991, Wagner, 1996). Care must therefore be exercised in applying data for individual reactions to metabolic activities of living cells as a whole.

1.3. The Interconversion of C_1-units and C_1-THF Synthase Activities

In most cells, because serine and glycine are the major sources of one-carbon units, entry to the active C_1 pool of intermediates is by way of 5,10-CH_2-H_4PteGlu$_n$. This folate derivative can participate directly in the synthesis of thymidylate (Reaction 9, Figure 1). Alternatively, 5,10-CH_2-H_4PteGlu$_n$ may be reduced to 5-CH_3-H_4PteGlu$_n$ (Reaction 11, Figure 1) for the biosynthesis of methionine, or oxidized to 10-HCO-H_4PteGlu$_n$ for use in

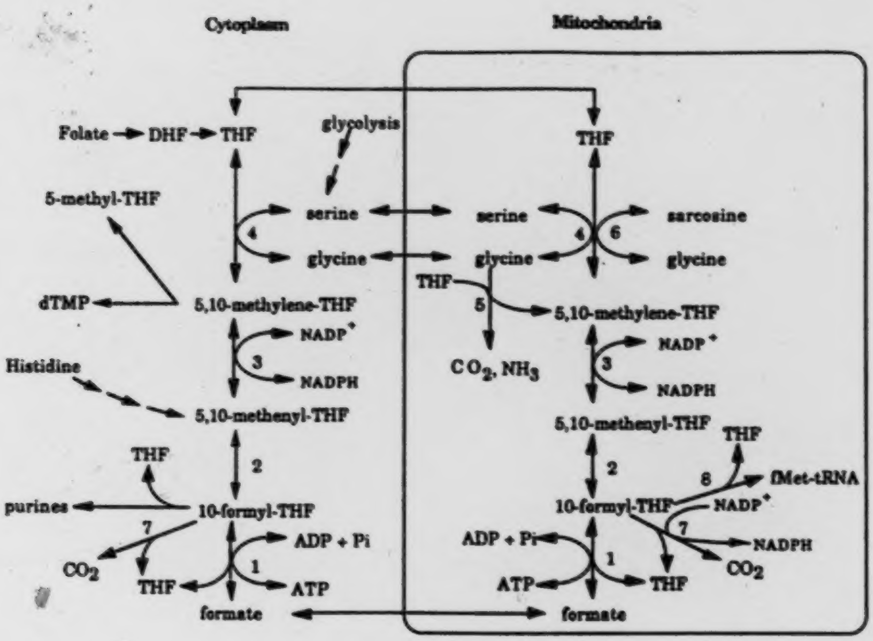

Cytoplasm
Mitochondria
Folate
DHF
THF
glycolysis
THF
5-methyl-THF
serine
serine
sarcosine
4
4
6
glycine
glycine
glycine
THF
dTMP
5,10-methylene-THF
5
5,10-methylene-THF
NADP+
NADP+
3
3
Histidine
NADPH
CO_2, NH_3
NADPH
5,10-methenyl-THF
5,10-methenyl-THF
THF
THF
2
2
8
fMet-tRNA
purines
10-formyl-THF
10-formyl-THF
NADP+
7
7
ADP + Pi
ADP + Pi
NADPH
CO_2
1
1
CO_2
THF
ATP
ATP
THF
formate
formate

Figure 4. *Compartmentation of Folate-mediated One-carbon Metabolism in Eukaryotes.*

The diagram is derived with some modifications from Appling (1991) for a mammalian and yeast cell system. The numbered reactions are: 1, 10-formyltetrahydrofolate synthetase; 2, 5,10-methenyltetrahydrofolate cyclohydrolase; 3, 5,10-methylenetetrahydrofolate dehydrogenase; 4, serine hydroxymethyltransferase; 5, glycine cleavage system; 6, sarcosine dehydrogenase (EC 1.5.99.1); 7, 10-formyltetrahydrofolate dehydrogenase; 8, methionyl-tRNA formyltransferase.

purine synthesis (MacKenzie, 1984; Appling, 1991). Many organisms, including plants, can also utilize formate as a one-carbon source (Cossins, 1987; Appling, 1991), and in these species the immediate product, 10-HCO-H_4PteGlu must be reduced prior to involvement in the synthesis of thymidylate, methionine, or serine. Thus, the interconversion of 5,10-CH_2-H_4PteGlu$_n$ and 10-HCO-H_4PteGlu$_n$ is of central importance in the utilization of one-carbon units (MacKenzie, 1984). This interconversion is achieved by three reversible reactions: 5,10-CH_2-H_4PteGlu dehydrogenase (DHY, Reaction 8, Figure 1), 5,10-CH^+-H_4PteGlu cyclohydrolase (CYC, Reaction 5, Figure 1), and 10-HCO-H_4PteGlu synthetase (SYN, Reaction 1, Figure 1; MacKenzie, 1984; Appling, 1991).

$$5,10\text{-CH}^+\text{-H}_4\text{PteGlu}_n + \text{NADPH} = 5,10\text{-CH}_2\text{-H}_4\text{PteGlu}_n + \text{NADP}$$

$$10\text{-HCO-H}_4\text{PteGlu}_n + \text{H}^+ = 5,10\text{-CH}^+\text{-H}_4\text{PteGlu}_n + \text{H}_2\text{O}$$

$$\text{Formate} + \text{H}_4\text{PteGlu}_n + \text{ATP} = 10\text{-HCO-H}_4\text{PteGlu}_n + \text{ADP} + \text{Pi}$$

In yeast and mammalian cells, DHY, CYC, and SYN activities are associated with a tri-functional protein (MacKenzie, 1984; Appling, 1991), commonly called C_1-tetrahydrofolate synthase or C_1-THF synthase (Rabinowitz, 1983; Appling and Rabinowitz, 1985a,b). In bacteria however, these activities are associated with a mono-functional SYN protein (Whitehead and Rabinowitz, 1988; Lovell et al., 1990) and with a bi-functional DHY-CYC complex (Dev and Harvey, 1978; Ljungdahl et al., 1980; D'Ari and Rabinowitz, 1991; Pawelek and MacKenzie, 1996). The structural organization and compartmentation of these proteins in plants is however not clear (Cossins, 1987; Appling, 1991). The following sections give a detailed review of the structural association of these activities in different species as well as their important role in one-carbon metabolism. However prior to this review, some recent studies on the degeneration of 10-HCO-H_4PteGlu$_n$ and the interconversion of 5-HCO-H_4PteGlu$_n$ and 5,10-CH^+-H_4PteGlu$_n$ are described as these have importance in folate metabolism.

1.3.1. The interconversion of 5-HCO-H_4PteGlu$_n$ and 5,10-CH^+-H_4PteGlu$_n$

5-HCO-H_4PteGlu, also known as folinic acid, has been proposed as a storage form of folates (MacKenzie, 1984). It is clear that whole organisms as well as cultured cells utilize this compound as a source of folate (MacKenzie, 1984). This folate is known to be formed from 5,10-CH^+-H_4PteGlu$_n$ by SHMT in the presence of glycine (Stover and Schirch, 1990), and is a strong inhibitor of bacterial, fungal and mammalian SHMT proteins (Kruschwitz et al., 1994; Stover and Schirch, 1991). There have been several recent studies of 5,10-CH^+-H_4PteGlu$_n$ synthetase (Reaction 7, Figure 1) as it represents a

$$5\text{-HCO-}H_4\text{PteGlu}_n + \text{Mg-ATP} = 5{,}10\text{-}CH^+\text{-}H_4\text{PteGlu}_n + \text{Mg-ADP} + P_i$$

metabolic route by which 5-HCO-H_4PteGlu$_n$ can participate in one-carbon metabolism (Stover et al., 1993a). As noted above, there is also evidence that the folate substrate of this irreversible reaction may be a regulator of one-carbon metabolism (Stover et al., 1993b). The synthetase has been purified to homogeneity from several eukaryotic and prokaryotic sources (Stover et al., 1993b), but to date, this enzyme has not been reported for plant species.

1.3.2. The degeneration of 10-HCO-H_4PteGlu$_n$

The enzyme 10-HCO-H_4PteGlu dehydrogenase (EC 1.5.1.6) catalyzes the NADP-dependent conversion of 10-HCO-H_4PteGlu to H_4PteGlu, and CO_2 (Equation 1).

$$\text{10-HCO-H}_4\text{PteGlu}_n + \text{NADP} \rightarrow \text{H}_4\text{PteGlu}_n + \text{CO}_2 + \text{NADPH} \qquad [1]$$

The purified hepatic proteins of pig (Rios-Orlandi et al., 1986; Min et al., 1988), rat (Scrutton and Beis, 1979; Min et al., 1988; Cook et al., 1991) and rabbit (Schirch et al., 1994) also exhibit lower levels of an NADP-independent hydrolase activity that converts 10-HCO-H_4PteGlu to H_4PteGlu and formate (Reaction 2). These proteins contain four

$$\text{10-HCO-H}_4\text{PteGlu}_n \rightarrow \text{H}_4\text{PteGlu}_n + \text{formate} \qquad [2]$$

identical 99-kDa subunits (Rios et al., 1986; Min et al, 1988; Cook et al., 1991; Schirch et al., 1994). Studies using proteolytic digestion of the enzyme showed that the N-terminal domain catalyzes the NADP-independent hydrolase activity and the C-terminal domain catalyzes the NADP-dependent aldehyde dehydrogenase activity (Cook et al., 1991; Schirch et al, 1994). However, to catalyze Equation 1, the two domains must be connected by a linker peptide (Schirch et al., 1994). The physiological importance of hydrolase and aldehyde dehydrogenase is not clear.

10-HCO-H_4PteGlu dehydrogenase represents about 0.5-1.2% of the soluble protein in mammalian liver (Cook et al., 1991, Kim et al., 1996), and binds tightly with its product H_4PteGlu$_5$ (Cook and Wagner, 1982; Min et al., 1988). This causes strong product inhibition to this enzyme (Kim et al., 1996). It follows that *in vivo*, where the intracellular concentration of 10-HCO-H_4PteGlu dehydrogenase exceeds that of

$H_4PteGlu_n$, most, if not all, of the folate pool in the form of $H_4PteGlu_n$ may be bound to this enzyme (Cichowicz and Shane, 1987; Horne et al., 1989; Strong and Schirch, 1989). To examine the availability of $H_4PteGlu_n$ for other folate-dependent reactions Kim et al. (1996) carried out *in vitro* studies. These workers incubated the dehydrogenase with an excess of either SHMT or C_1-THF synthase. Under these conditions $H_4PteGlu_5$, arising during the dehydrogenase reaction, was released and product inhibition was abolished. The data suggest that the bound folate was rapidly transferred to the other folate-dependent enzymes.

The physiological function of 10-HCO-H_4PteGlu dehydrogenase is not clear. It has been proposed that it represents an important site for binding hepatic folylpolyglutamates and for regulating the interconversion of 10-HCO-$H_4PteGlu_n$ and $H_4PteGlu_n$ (Krebs et al., 1976; Champion et al., 1994). Neymeyer and Tephly (1994) have also suggested that 10-HCO-H_4PteGlu dehydrogenase may play an important role in removing formate formed during methanol toxicity.

1.3.3. The C_1-THF synthase activities of mammalian cells

A homogeneous protein, isolated from sheep liver, was first reported by Paukert et al. (1976) that catalyzed all three reactions of C_1-THF synthase namely, SYN, CYC and DHY, at high specific activity. This protein contained a single type of constituent polypeptide (homodimeric with a subunit M_r = 108,000) and it was proposed to call the enzyme formyl-methenyl-methylene-tetrahydrofolate synthetase-(combined) (Paukert et al., 1976). Later studies demonstrated that this tri-functional protein occurred widely in mammalian and yeast cells (MacKenzie, 1984; Appling, 1991). Appling and Rabinowitz (1985a and 1985b) called this multi-functional protein C_1-tetrahydrofolate (C_1-THF) synthase, a term that is now generally accepted.

cDNAs encoding C_1-THF synthase have been cloned and sequenced from human (Hum et al., 1988) and rat (Thigpen et al., 1990) tissues. Both nucleotide sequences have an open reading frame that encodes a protein of 935 amino acids. This protein shows extensive homology within mammalian species and with the analogous proteins of bacteria and yeast (Hum et al., 1988; Thigpen et al, 1990). The absence of a mitochondrial targeting sequence in both proteins indicated that the cDNA encoded a cytosolic form of the enzyme. Studies of steady-state levels of mRNA and levels of the individual enzyme activities in rat tissues suggest that this gene is expressed in a tissue-specific manner with regulation being predominantly by pretranslational events (Thigpen et al., 1990).

Considerable research effort has been applied to the investigation of the SYN, CYC and DHY domains of this protein. As a result, analysis of proteolytic fragments of rabbit liver C_1-THF synthase has shown that the N-terminal portion contains the DHY-CYC activities while a large fragment, that includes the carboxyl-terminus, catalyzes SYN activity (Villar et al., 1985). Further study of this tri-functional protein by differential scanning calorimetry showed that the native enzyme contains two protein domains which are related to the two protein fragments generated by proteolysis (Villar et al., 1985). The larger of the two domains contains the active site for SYN activity while the smaller domain contains the active site of the DHY and CYC reactions. These two domains have little or no interaction with each other with respect to either structural or catalytic properties of the native enzyme (Villar et al., 1985). However, the DHY-CYC activities of the enzyme from pig liver are not kinetically independent (Drummond et al., 1983), and appear to share a common folate-binding site (Smith and MacKenzie, 1985). In another study, the DHY-CYC and SYN domains of human C_1-THF synthase have been successfully expressed in *E. coli* with significantly higher specific activities than shown by the native hepatic form of the protein (Hum and MacKenzie, 1991).

In other work, Barlowe and Appling (1988) provided strong evidence that in rat liver, serine and glycine are degraded in the mitochondria by the combined action of mitochondrial SHMT, GDC, and mitochondrial C_1-THF synthase. The authors concluded that formate, after being transported to the cytosol could participate as a one-carbon source in purine biosynthesis. Support for this notion was derived from a separate study on SHMT and C_1-THF synthase in a murine leukemia cell line L1210. In this work, Strong et al. (1990) found that the complete inhibition of cytosolic SHMT would neither significantly decrease the rates of purine and thymidylate biosynthesis nor significantly alter the rate of interconversion of tetrahydrofolate cofactors to dihydrofolate with subsequent inhibition of dihydrofolate reductase. These results suggest that the SYN, CYC and DHY activities of mammalian mitochondria have important roles in the generation of one-carbon units that are subsequently used in folate-dependent reactions in cytosol.

It is now clear that the DHY activity of C_1-THF synthase is exclusively NADP-dependent (MacKenzie, 1984; Appling, 1991). In addition, a unique NAD-dependent DHY is expressed in transformed and developmental tissues of normal adult mammals

(Mejia and MacKenzie, 1985). This latter homodimeric bi-functional protein (subunit Mr = 34,000), also contains CYC, and has been purified to homogeneity from *Ehrlich* ascites tumor cells (Mejia et al., 1986). Like C_1-THF synthase, the NAD-dependent protein channels folate intermediates between the DHY and CYC sites. This channeling is not affected by the number of glutamyl residues in the folate substrate (Rios-Orlandi and MacKenzie, 1988).

Studies of murine tumor cells, involving subcellular fractionations and Western blot analyses, have shown that the NAD-dependent dehydrogenase activity is predominantly mitochondrial, while the NADP-dependent enzyme of these cells is cytosolic (Mejia and MacKenzie, 1988). The mitochondrial nature of this bi-functional protein was also confirmed by sequencing the appropriate cDNA clones of mouse (Belanger and MacKenzie, 1989) and human (Peri et al., 1989) tissues. The murine nucleotide sequence encodes a 350 amino acid protein which contains a putative mitochondrial-targeting signal peptide (Belanger and MacKenzie, 1989). This amino acid sequence shows extensive homology with the corresponding amino-terminal sequence of the C_1-THF synthase from human cells, yeast cytosol, and yeast mitochondria (Belanger and MacKenzie, 1989). The mitochondrial targeting peptide in the human enzyme is shorter than that of the mouse enzyme, but the mature proteins are 95% identical (Peri et al., 1989). In contrast, a recent study of insect cells revealed a cytosolic NAD-dependent, DHY-CYC complex (Tremblay et al., 1992). Neither NADP-dependent C_1-THF synthase nor SYN activity are expressed in these insect cell lines.

1.3.4. The C_1-THF synthases of yeast cells

Yeast (*Saccharomyces cerevisiae*) C_1-THF synthase occurs as a cytosolic protein encoded by the *ade-3* gene (Appling and Rabinowitz, 1985b; Staben and Rabinowitz, 1986; Barlowe and Appling, 1990; Song and Rabinowitz, 1993) and as a mitochondrial protein encoded by the *MIS 1* gene (Shannon and Rabinowitz, 1986 and 1988; Appling, 1991). Both of these genes have been cloned and sequenced by Rabinowitz's group (Staben and Rabinowitz, 1986; Shannon and Rabinowitz, 1988).

Chemical treatments and immunotitration studies of the cytosolic protein provided evidence for an overlapping CYC:DHY site that was independent of the SYN active site (Appling and Rabinowitz, 1985b). The affinity of $H_4PteGlu_n$ for the SYN site increases with polyglutamate chain length (Rabinowitz, 1983). In contrast, the CYC and DHY reactions do not show a marked preference for polyglutamate substrates (Rabinowitz, 1983). By using site-directed mutagenesis, Kirksey and Appling (1996) found that the Asp^{449}, a highly conserved aspartate in the putative 10-HCO-$H_4PteGlu_n$ binding site of the cytosolic C_1-THF synthase, is not a critical catalytic residue for synthetase activity. MacKenzie (1984) has reviewed earlier work on the catalytic and physical properties of this tri-functional protein including the channeling of folates between the CYC and DHY reactions. The yeast *MIS 1* gene (Shannon and Rabinowitz, 1988) has an open reading frame for a protein of M_r = 106,235 that includes a nucleotide sequence (residues 1-34) for a putative mitochondrial signal peptide. Without this leader sequence, the "mature" protein had a mass in agreement with that determined by electrophoresis (Shannon and

Rabinowitz, 1986 and 1988). Although the cytosolic and mitochondrial C_1-THF synthase proteins are encoded by two different nuclear genes, immunotitration experiments and amino acid sequence analysis suggest that these two isozymes are structurally related (Shannon and Rabinowitz, 1986 and 1988).

The roles of yeast C_1-THF synthase isoenzymes in one-carbon metabolism have also been extensively examined. Theoretically, 10-HCO-H_4PteGlu$_n$ needed for cytoplasmic purine biosynthesis might arise from 5,10-CH_2-H_4PteGlu$_n$ or from formate. The metabolic origins of this formate might include the mitochondrial degradation of sarcosine or serine to 10-HCO-H_4PteGlu via the intermediary formation of 5,10-CH_2-H_4PteGlu$_n$ (Appling, 1991). Conceivably, the mitochondrial isoenzyme, by reversal of its 10-HCO-H_4PteGlu synthetase activity, might produce formate for export to the cytoplasm. To assess the importance of this mitochondrial pathway, Shannon & Rabinowitz (1988) disrupted the *MIS 1* gene *in vitro* and introduced copies of the non-functional gene into the yeast chromosome. The resulting mutants grew on a simple medium containing a non-fermentable carbon source suggesting that the mitochondrial form of C_1-THF synthase was not essential for high rates of purine synthesis or for the initiation of mitochondrial protein synthesis. On the other hand, deletion of the *ade-3* gene resulted in an absolute requirement for adenine. However, point mutations that inactivated all three activities of C_1-THF synthase did not result in adenine auxotrophy. This raises the possibility that the synthase protein may be a structural component of a metabolon for purine biosynthesis in *Saccharomyces* (West et al., 1996). These data also imply that 10-HCO-H_4PteGlu$_n$, needed for purine synthesis may be derived by a cytosolic pathway involving a mono-functional NAD-dependent 5,10-CH_2-H_4PteGlu$_n$ dehydrogenase (Barlowe and Appling, 1990; West et al, 1993 and 1996) Support for this contention and for catalytic and non-catalytic roles for the soluble C_1-THF synthase have recently been reported by Appling's group (West et al., 1996).

The role of C_1-THF synthase has also been assessed using ^{13}C-labeled formate. Yeast cells carrying genetic deletions for either the cytoplasmic or the mitochondrial isoenzyme used formate for serine synthesis which occurred primarily in the cytosol (Pasternack et al., 1992 and 1994b). Strains expressing only the mitochondrial isoenzyme produced [2-^{13}C]glycine and [3-^{13}C]serine from [^{13}C]formate. Thus one-carbon units generated from formate by both forms of the synthase, were reduced to 5,10-CH_2-H_4PteGlu$_n$ prior to incorporation into glycine and serine. Detailed ^{13}C NMR analysis of purine synthesis showed that the mitochondrial isoenzyme also had an important role in generating formate (Pasternack et al., 1994a). Approximately 25% of this formate was transported to the cytosol where it was incorporated into purines via 10-HCO-H_4PteGlu$_n$. These workers have also applied ^{13}C NMR methods to examine the metabolic fates of several other one-carbon donors in yeast (Pasternack et al., 1996).

1.3.5. The individual activities of C_1-THF synthase in bacterial cells

The *C. acidiurici* and *C. thermoacetium* nucleotide sequences that encode a mono-functional SYN protein have been sequenced, and have calculated molecular weights of 59,599 Da and 59,983 Da respectively (Whitehead and Rabinowitz, 1988; Lovell et al., 1990). Of the 556 amino acid residues shared between the two *Clostridium* sequences, 66.4% are identical (Lovell et al., 1990). Interestingly, both sequences show 47-48% identity with the synthetase domain of yeast cytosolic C_1-THF synthase (Whitehead and Rabinowitz, 1988; Lovell et al., 1990). This is in agreement with the earlier study of the immunological cross-reactivity of the yeast cytosolic C_1-THF synthase and the SYN protein of *C. acidiurici* (Staben and Rabinowitz, 1983). In recent studies, $[^{18}O]H_2O$ incorporation combined with ^{13}C NMR spectroscopy and site-directed mutagenesis have been used to study the catalytic properties of *C. cylindrosporum* SYN (Song et al., 1993; Kounga et al., 1996).

Escherichia coli (Dev and Harvey, 1978; D'Ari and Rabinowitz, 1991), *Clostridium thermoaceticum* (Ljungdahl et al., 1980) and *Photobacterium phosphoreum* (Pawelek and MacKenzie, 1996) express a bi-functional DHY-CYC complex that lacks SYN activity. The DHY activity of these proteins is NADP-dependent except that of *P. phosphoreum* which uses both NADP and NAD as a cofactor. In addition, mono-functional NAD- or NADP-dependent DHY have been purified from *Acetobacterium woodii* (Tanner et al., 1978) and *Clostridium formicoacetium* (Ljungdahl et al., 1980).

The nucleotide sequences encoding the bi-functional DHY-CYC protein have been isolated and sequenced from *Escherichia coli* (D'Ari and Rabinowitz, 1991) and *Photobacterium phosphoreum* (Pawelek and MacKenzie, 1996). The derived amino acid sequence of the *E. coli* enzyme shows 50% identity with the human and mouse bi-functional NAD-dependent mitochondrial enzymes, and 40-45% identity with the DHY-CYC domains of eukaryotic C_1-THF synthases (D'Ari and Rabinowitz, 1991). Like the *E. coli* protein, the *P. phosphoreum* amino acid sequence shares a higher degree of similarity with the eukaryotic mitochondrial bi-functional DHY-CYC than with the DHY-CYC domains of eukaroytic C_1-THF synthase (Pawelek and MacKenzie, 1996).

1.3.6. C_1-THF synthase activities of higher plants: the present studies

The structural organization and compartmentation of SYN, CYC and DHY activities in plants is less certain than outlined above for other eukaryotes. Early studies showed that many plant tissues contain significant levels of these activities (for review see Cossins, 1980). However, the majority of these investigations did not employ extraction buffers that contained protease inhibitors. As a result, the existence of tri-functional C_1-THF synthase complexes in higher plants was not thoroughly examined until the present decade. In spinach leaves (Nour and Rabinowitz, 1991) and pea cotyledons (Kirk et al., 1994), it is clear that SYN activity is associated with a protein that lacks DHY and CYC activities. The SYN protein of pea cotyledons occurs principally as a cytosolic homodimer that uses $H_4PteGlu_n$ as preferred folate substrates (Kirk et al., 1994). The spinach leaf SYN has structural homologies to the SYN domains of mammalian and yeast C_1-THF synthase and displays a putative sequence for the binding of polyglutamyl folates (Nour and Rabinowitz, 1992). However, until the current studies were undertaken, the structural organization and subcellular distribution of these three activities, especially DHY and CYC, in plants were not clear. Furthermore, there had also been no reports of the cloning or sequencing of plant genes encoding DHY or CYC proteins.

Recent studies of photosynthetic tissues have provided new insights on their roles in folate metabolism and biosynthesis. For example, leaf mitochondria contain $H_4PteGlu_n$

derivatives (Besson et al., 1993) and key folate-dependent enzymes including SHMT, GDC, DHFR and TS (Neuburger et al., 1996). In addition, these mitochondria contain important enzymes of a folate biosynthetic pathway that conceivably forms $H_4PteGlu_n$ from pteridine precursors (Neuburger et al., 1996). Although it is not clear if this pathway generates all of the $H_4PteGlu_n$ needed to support cellular one-carbon metabolism there is good evidence that leaf mitochondria may be a major site for dihydropteroate biosynthesis in plant cells (Rebeille et al., 1997). In other earlier studies, it was reported that the total folate pool was enlarged when etiolated leaves were exposed to light (Spronk and Cossins, 1972). Light also increases the specific activities of SYN, DHY (Gifford and Cossins, 1982), SHMT and GDC (Turner et al., 1993; Walker and Oliver, 1986; Oliver and Raman, 1995) in the leaves of several plant species. Furthermore, greening in the presence of α-hydroxy-2-pyridinemethane sulfonate, an inhibitor of glycollate oxidation, reduced this enhancement of SYN activity in barley leaves as well as the ability to metabolize labeled formate. Thus formate for the SYN reaction in barley leaves may be partly derived from glycollate during photorespiration (Gifford and Cossins, 1982). Conceivably, leaf mitochondria, like those of non-green tissues (Cossins, 1987), also contain enzymes that oxidize part of the 5,10-CH_2-$H_4PteGlu_n$ arising during the photorespiratory cleavage of glycine. There is still little information on this aspect of plant folate metabolism.

The other major metabolic compartment of leaves, chloroplasts, contain a folate pool that is utilized in the transformylation of methionyl-tRNA (Staben and Rabinowitz, 1984; Cossins 1987). There is also evidence that isolated pea chloroplasts convert formate to serine (Shingles et al., 1984) and have SHMT activity (Cossins, 1980). It therefore follows that chloroplasts contain C_1-THF synthase activities for the generation and interconversion of one-carbon units needed in these reactions. However to date, basically no information is available to assess these possibilities.

The present thesis research has therefore examined the subcellular distribution and structural organization of SYN, DHY and CYC activities in the actively growing leaves of pea seedlings. The cytosolic forms of these proteins, which accounted for the bulk of extracted activities, have been purified to homogeneity in the presence of the protease inhibitor PMSF. Antibodies raised against the purified proteins were then used to examine mitochondrial and chloroplast extracts for the presence of immunologically related proteins. Also, by using immunoaffinity chromatography, the possible structural organization of these activities in mitochondrial extracts was examined. In addition, the effect of greening on the DHY, CYC and SYN activities was investigated by examining changes in enzyme activities and the levels of cross-reacting proteins. The possible structural association of these enzymes in other plants and in fungal species was also examined. Furthermore, by immunoscreening pea leaf λgt11 libraries, a cDNA encoding a bi-functional DHY-CYC protein was isolated and sequenced.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Reagent-grade chemicals were purchased either from Sigma Chemical Co., Fisher Scientific, or British Drug House (BDH Chemicals). More specialized biochemicals, substrates and analytic reagents were obtained from the following sources. PteGlu, (6R, S)-H_4PteGlu, heparin agarose, Sephacryl S-200 and S-300, and goat anti-rabbit IgG linked to alkaline phosphatase were supplied by Sigma. Matrex Green A and Matrex Orange A were purchased from Amicon; Sephadex G-75 was obtained from Pharmacia. Bio-Gel P-6, hydroxyapatite, Silver Stain Kits, Bradford's reagent, Affi-Gel Hz Hydrazide Gel, nitrocellulose membranes and Zeta-Probe blotting membranes were from Bio-Rad. DEAE-52 cellulose was obtained from Whatman. Chemiluminescence Western blot kits were supplied by Boehringer Mannheim and *E. coli* strain Y1090r$^-$ was from Stratagene. ECL™ immunoscreening reagent, Hybond™ C-extra nitrocellulose filters, Hybond-N$^+$ nylon filters and ^{32}P-dCTP were purchased from Amersham Life Science. Biotrans$^+$ nylon membranes were supplied by ICN Biomedical, Inc. RNA markers, RNAgents Total RNA Isolation System and PolyATRACT mRNA Isolation System were purchased from Promega. Restriction endonucleases, Taq DNA polymerase and Klenow fragment of DNA polymerase I were supplied either from Pharmacia, Bethesda Research Laboratories (BRL) Inc., New England BioLabs, or Boehringer Mannheim. Primers used in PCR and DNA sequencing were prepared by the DNA Synthesis Laboratory, Department of Biological Sciences, University of Alberta. λgt11 expression libraries, constructed using

10 d light-grown or 9 d dark-grown pea leaves were donated by Dr. David Macherel, Physiologie Cellulaire Vegetale, Centre d'Etude Nucleaires, Grenoble, France. Polyclonal antibodies were raised in rabbits by staff of the Animal Services Laboratory, University of Alberta.

2.2. *Plant Materials*

Seeds of pea (*Pisum sativum* L. cv. Homesteader), bean (*Phaselous vulgaris* L. cv. Bountiful), barley (*Hordeum vulgare* L. cv. Galt), wheat (*Triticum aestivum* L. cv. Neapawa) and corn (*Zea mays* L. var. Gills Early Market) were supplied by Apache Seeds Ltd., Edmonton, Alberta. Plant seedlings were raised in growth chambers (16 h days, light intensity of 700 μeinsteins m^{-2} sec^{-1}, 25 °C; 8 h nights, 20 °C, 50% R.H.) for 14 d as described by Imeson et al. (1990). Leaves of etiolated pea seedlings, grown under these conditions but in darkness for 9 d, were harvested in subdued light (15 watt fluorescent bulb, Green No. 7 Kodak filter). Leaf extracts were prepared immediately after excision.

2.3. *Assay of Enzyme Activities*

10-HCO-H_4PteGlu synthetase (EC 6.3.4.3)

$$HCOOH + H_4PteGlu + ATP \Leftrightarrow 10\text{-}HCO\text{-}H_4PteGlu + ADP + P_i$$

10-HCO-H_4PteGlu synthetase was assayed spectrophotometrically (Appling and Rabinowitz, 1985) using the reaction conditions of Kirk et al. (1994). The standard reaction system (1 ml) contained 100 μmol triethanolamine HCl buffer (pH 7.5), 100 μmol ammonium formate, 2.5 µmol $MgCl_2$, 200 µmol KCl, 1 µmol (6R,S)-5,10-H_4PteGlu, 2 µmol ATP and up to 50 µl of plant extract. After incubation at 30 °C for 5-15 min, the reaction was terminated by addition of 2 ml of 0.36 M HCl and A was read at 350 nm. Product formation is expressed in nmol min^{-1} based on an extinction coefficient of 24,900 M^{-1} cm^{-1} (de Mata and Rabinowitz, 1980).

5,10-CH^+-H_4PteGlu cyclohydrolase (EC 3.5.4.9)

$$5,10\text{-}CH^+\text{-}H_4PteGlu + H_2O \Leftrightarrow 10\text{-}HCO\text{-}H_4PteGlu$$

5,10-CH^+-H_4PteGlu cyclohydrolase was measured by changes in A at 355 nm (de Mata and Rabinowitz, 1980). The substrate, (6R,S)-5,10-CH^+-H_4PteGlu, was prepared by dissolving 15 µmol Ca-leucovorin in 9.64 ml 0.1 N HCl with the addition of 0.36 ml β-mercaptoenthanol. The mixture was then incubated in darkness in a vacuum desiccator at 25 °C for 24 h before being stored in darkness at 4 °C. The reaction system (1 ml), containing 200 µmol K maleate (pH 8), 75 nmol (6R,S)-5,10-CH^+-H_4PteGlu, 100 µmol β-mercaptoethanol and up to 100 µl of enzyme extract was incubated at 20 °C. After correction for non-enzymatic substrate hydrolysis, reaction rates were calculated using the above extinction coefficient, and expressed as substrate hydrolyzed (nmol min^{-1}).

NADP-dependent 5,10-CH_2-H_4PteGlu dehydrogenase (EC 1.5.1.5)

$$5,10\text{-}CH_2\text{-}H_4PteGlu + NADP \Leftrightarrow 5,10\text{-}CH^+\text{-}H_4PteGlu + NADPH + H^+$$

NADP-dependent 5,10-CH_2-H_4PteGlu dehydrogenase was assayed spectrophotometrically (Appling and Rabinowitz, 1985) using 1 ml reaction systems containing 25 μmol BES buffer (pH 6.5), 1 μmol (6R,S)-H_4PteGlu, 10 μmol HCHO, 0.6 μmol NADP, 100 μmol KCl and up to 50 μl plant extract. After incubation at 37 °C for 5-15 min, 2 ml of 1 M HCl were added and 5,10-CH^+-H_4PteGlu was measured at 350 nm using the above extinction coefficient. Activity was expressed as product formed (nmol min^{-1}).

NAD-dependent 5,10-CH_2-H_4PteGlu dehydrogenase (EC 1.5.1.15)

$$5,10\text{-}CH_2\text{-}H_4\text{PteGlu} + \text{NAD} \Leftrightarrow 5,10\text{-}CH^+\text{-}H_4\text{PteGlu} + \text{NADH} + H^+$$

NAD-dependent 5,10-CH_2-H_4PteGlu dehydrogenase was assayed spectrophotometrically (Mejia and MacKenzie, 1985) using 1 ml reaction systems containing 100 μmol K-maleate buffer (pH 8.0), 2.5 μmol (6R,S)-H_4PteGlu, 10 nmol HCHO, 0.5 μmol NAD, 200 μmol KCl and up to 50 μl plant extract. After incubation at 30 °C for 15 min, 2 ml of 1 M HCl were added and 5,10-CH^+-H_4PteGlu was measured at 350 nm using the above extinction coefficient. Activity was expressed as product formed (nmol min^{-1}).

Other Enzyme Assays

Alcohol dehydrogenase and succinate dehydrogenase were assayed as marker enzymes of cytosolic and mitochondrial fractions respectively. Both activities were measured by standard procedures (Coffin and Cossins, 1986). Phosphoribulokinase was assayed as a chloroplast marker enzyme (Kagawa, 1982).

2.4. Organelle Isolation and Preparation of Enzyme Extracts

The preparation of leaf extracts and the isolation of purified mitochondria on Percoll gradients were according to Douce et al. (1987). The 12,000 x g supernatant fraction, produced by differential centrifugation of these extracts (Douce et al., 1987), was used as a source of cytosolic proteins. Pea leaf chloroplasts were isolated on Percoll gradients according to Schuler and Zielinski (1989). To prepare whole chloroplast lysate, the intact chloroplasts were resuspended in chloroplast lysis buffer (62.5 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 1 mM PMSF) and incubated on ice for 15 min with occasional vortexing. After centrifuging the whole chloroplast lysate for 5 min in an Eppendorf microfuge operated at full speed, the supernatant was saved as the stromal fraction and the pellet was designated the thylakoid membrane fraction (Schuler and Zielinski, 1989). The biochemical purities of the isolated organelles were assessed by assay of the above mentioned marker enzymes.

Mitochondria and chloroplasts (including stromal and thylakoid membrane fractions) were resuspended in 25 mM HEPES buffer (pH 7.5) containing 0.1% Triton X-100, 1 mM PMSF, 10 mM β-mercaptoethanol, 10 mM KCl, and 20% glycerol (v/v). These suspensions were then sonicated (2 pulses of 1.5 minutes at 4 °C) using a ultrasonic homogenizer (Cole-Parmer Instrument Co., 4710 series) with the output control set at 70. The resulting extracts were used in enzyme assays, immunoblots and for immunoaffinity chromatography.

2.5. Purification of Cytosolic 5,10-CH_2-H_4PteGlu Dehydrogenase and 5,10-CH^+-H_4PteGlu Cyclohydrolase

The aerial shoots of 14 d pea seedlings were homogenized using a domestic Moulinex blender in 50 mM Tris-HCl (pH 7.5), containing 25% (v/v) glycerol, 10 mM β-mercaptoethanol and 1 mM PMSF (Buffer A). The resulting homogenate (Step 1 protein) was centrifuged (10,000 x g, 20 min), treated with streptomycin sulfate (final concentration of 1%, w/v; Step 2 protein), $(NH_4)_2SO_4$ (pellet of 55-75% saturation range; Step 3 protein) and chromatographed on heparin agarose (Kirk et al., 1995) to yield Step 4 protein. After concentrating the protein by ultrafiltration (Amicon Centriprep-10) followed by desalting using Bio-Gel P-6, the extracts were chromatographed on Matrex Green A (Kirk et al., 1995) to give Step 5 protein. Enzyme-active fractions were pooled, concentrated by ultrafiltration and applied to a 3 x 80 cm column of Sephadex G-75. Protein was eluted with Buffer A containing 10 mM Tris-HCl. Fractions containing DHY and CYC activities were combined, concentrated by ultrafiltration and applied to a 1.5 X 6 cm column of Matrex Orange A that had been pre-equilibrated in Buffer A containing 10 mM Tris-HCl. The column was washed with the loading buffer (25 ml) and DHY-CYC protein was eluted with a linear KCl gradient (0.05 M to 0.4 M in the loading buffer).

For molecular weight determinations by gel filtration, bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa) and aprotinin (6.5 kDa) were used as molecular weight standards. Blue dextran (2,000 kDa) was used to determine V_o. Proteins including DHY-CYC were separately applied to a 2.5 x 80 cm column of Sephadex G-75 column that had been previously equilibrated with 100 mM KH_2PO_4 buffer (pH 7) containing 25% (v/v) glycerol, 10 mM β-mercaptoethanol and 1 mM PMSF. Fractions of 3 ml were collected at a flow rate of 20 ml hour^{-1} and measurement of A_{280} was used to locate the elution position of each protein.

2.6. Purification of Cytosolic 10-HCO-H_4PteGlu Synthetase from Pea Leaf Extracts

The preparation of the initial homogenate (Step 1 protein) and its subsequent fractionation with $(NH_4)_2SO_4$ were carried out as described by Nour and Rabinowitz (1991). The resulting 50-70% $(NH_4)_2SO_4$ fraction (Step 2 protein) was dissolved in 25 mM HEPES buffer (pH 7.5), containing 1 mM PMSF, 10 mM 2-mercaptoethanol, 10 mM KCl, and 20% (v/v) glycerol (Buffer B). The extract was desalted by passing through columns of Bio-Gel P-6 and applied to a 5 x 20 cm column of DEAE-52 cellulose pre-equilibrated with Buffer B. The column was washed with 2.5-3 liters of Buffer B until the A_{280} of the wash was negligible. SYN activity was eluted using a KCl linear gradient (0.01 M to 0.3 M) in loading buffer to give Step 3 protein. The combined SYN fractions were concentrated by ultrafiltration and diluted in Buffer B to give a final KCl concentration of 50 mM before loading onto a heparin agarose column (2.5 x 6 cm) pre-equilibrated with Buffer B containing 50 mM KCl. The column was washed with 200 ml of the pre-equilibration buffer and SYN was eluted with a linear gradient of 50 to 500 mM KCl in 400 ml loading buffer. Fractions containing SYN activity were pooled, concentrated by ultrafiltration and chromatographed on hydroxyapatite as described by Nour and Rabinowitz (1991).

A 3 x 80 cm column of Sephacryl S-300 was employed to determine the native molecular weight of 10-HCO-H_4PteGlu synthetase. The column was calibrated using cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa). Blue dextran (2,000 kDa) was used to determine V_o. Column chromatography and the detection of protein peaks were carried out as described above (Section 2.6), with the exception that Buffer B was used for protein elution.

2.7 Preparation of Polyclonal Antibodies and ELISA

Samples of purified DHY-CYC and SYN were lyophilized and used for the preparation of polyclonal antibodies in rabbits (Tijssen, 1985). For the first injection, about 85 μg of the purified protein was dissolved in 0.8 ml of 0.8% (w/v) sterile saline and homogenized to an emulsified state with 0.8 ml of Freund's complete adjuvant. After 1 month, an injection of 45 μg of purified protein in Freund's incomplete adjuvant (FIA) was administered. One month later, a test bleed was done to check the titer of antisera. If this was satisfactory, the rabbits received a final boost injection of 45 μg of protein in FIA. Serum was collected after 1 week. IgG was partially purified by Na_2SO_4 precipitation and DEAE-cellulose column chromatography (Johnstone and Thorpe, 1987) before being lyophilized and stored at -20 °C.

For the detection of antiserum titers by ELISA, microtiter plates were coated with purified DHY-CYC or SYN, washed with PBST buffer (pH 7.5), blocked with 1% BSA and incubated with dilutions of antisera (Tijssen, 1985). The wells were then washed with PBS buffer and incubated with a goat anti-rabbit IgG (whole molecule) conjugated to alkaline phosphatase (Sigma). Absorbance at 405 nm was measured using a Bio-Rad ELISA reader after the addition of p-nitrophenyl phosphate.

To test the cross-reactivity of DHY-CYC-Ab and SYN-Ab with proteins in mitochondrial extracts and leaf extracts from other plant species, indirect competitive ELISA assays were conducted. The same protocol was followed as for antiserum titers (see above), except that after the microtiter plates were coated with antigen (homogeneous DHY-CYC or SYN protein) and blocked with BSA, equal volumes of test proteins (in serial dilutions with PBST, pH 7.2) and DHY-CYC-Ab or SYN-Ab were added to the wells. Control wells lacked the plant test extract which was replaced by PBST. Incubation with the secondary antibody and absorbance measurement were described as above.

2.8. Immunoaffinity Chromatography of Mitochondrial Extracts

Partially purified DHY-CYC-Ab (20 mg) were coupled, with approximately 60% efficiency, to 10 ml of Bio-Rad Affi-Gel Hz Hydrazide Gel according to the manufacturer's manual. 5 ml of gel, in a Bio-Rad Econo column, were pre-equilibrated with Buffer B as described in Section 2.6 and mitochondrial extract (ca. 80 mg protein) was applied at room temperature. Unbound protein was collected in the column wash and assayed for enzyme activity. The column was then washed with 15 ml of 1 M GuHCl followed by 30 ml of 0.2 M Gly-HCl buffer (pH 2.8). The collected fractions of 1 ml each were immediately neutralized to pH 7.5 by the addition of 1 M KOH. These fractions were then assayed for enzyme activity. Aliquots of each fraction were also subjected to immunoblot analyses.

2.9. SDS-PAGE and Western Blot Analyses

Protein was assayed by the method of Bradford (1976). SDS-PAGE and Western blot transfers were carried out using a Bio-Rad Mini Protein II system. Electrophoreses were accomplished on 4% stacking and 12 % separating SDS-PAGE slab gels according to the manufacturer's manual. Prior to electrophoresis, all protein samples were denatured by mixing with 4 volumes of sample buffer (0.25 M Tris-HCl, pH 6.8; 10% glycerol; 2% SDS; 5% β-mercaptoethanol; and 0.00125% bromphenol blue) and boiling for 5 minutes. Protein bands were detected by silver staining according to the Bio-Rad instruction manual.

Bio-Rad nitrocellulose membranes were used in Western blot transfers with the supplier's protocol being followed. Western blot immuno-detections employed chemiluminescence blotting substrate kits supplied by Boehringer Mannheim.

2.10. Isolation of DNA and RNA

Pea genomic DNA was isolated from leaves of 14 d plants by following the CTAB (hexadecyltrimethylammonium bromide) method described by Doyle and Doyle (1990). DNA was spectrophotometrically quantified by absorbance readings at 260 nm. The quality of the recovered DNA was monitored by reference to the A_{260}/A_{280} ratio (Ausubel et al., 1992).

For the isolation of RNA, plant tissues were frozen in liquid nitrogen immediately after harvest and stored at -70 °C until analyzed. All plastic tubes and pipette tips were purchased as sterile or RNase-free from Corning Costar Inc. Solutions used in RNA isolation were all treated overnight with DEPC (final concentration 0.1%, v/v) at room temperature, and then autoclaved for 30 minutes to remove traces of DEPC (Wilkinson, 1991). Total RNA was isolated using the Promega RNAgents Total RNA Isolation System. The manufacturer's technical manual (No. 087) provided the necessary protocol. Pellets of RNA, precipitated by ethanol, were dissolved in nuclease-free water, spectrophotometrically quantified at 260 nm (Wilkinson, 1991) and stored at -20 °C.

2. 11. Synthesis of Primers and DNA Sequencing

Primer syntheses and DNA sequencing were carried out by the DNA Synthesis Laboratory, Department of Biological Sciences, University of Alberta. Primers were synthesized on a 391 DNA Synthesizer PCR-MATE (ABI Applied Biosystems) and DNA sequencing employed an ABI Applied Biosystems 373 DNA Sequencer STRETCH. Table 1 lists the DNA primers used in the present work.

2.12. Oligolabeling and Restriction Enzyme Digestion of DNA

DNA (32 µl) was denatured by boiling for 5 min in a water bath and immediately cooling in a salt-ice bath for 3 min. 10 µl of OLB (oligolabeling buffer, containing 0.25 M Tris-HCl, pH 7.5; 50 mM $MgCl_2$; 150 mM β-mercaptoethanol; 0.3 mM each of dATP, dGTP and dTTP; 20 U/ml Random Primers) and 5 µl of α-^{32}P-dCTP (10 µCi/µl) were then added to the reaction tube. The labeling reaction was started by the addition of 1 µl of Large Fragment of DNA Polymerase I (Klenow Fragment, 4.3 U/µl), and incubation was at 37 °C for 1-2 h. The oligolabeled DNA was then denatured (see above) immediately before being used as a probe in hybridization reactions.

For restriction enzyme digestion, DNA solutions were adjusted to 45 µl with sterilized dH_2O in a sterile Eppendorf tube and then mixed with 5 µl of 10X GIBCO-BRL REact buffer. Digestion was started by addition of the appropriate restriction endonuclease (1-5 µl , 5 U/µg DNA). The tube was vortexed and centrifuged briefly, and then incubated at 37 °C overnight (Ausubel et al., 1992).

2.13. Polymerase Chain Reaction (PCR)

For sequence analysis, Taq PCR was primarily utilized to amplify DNA fragment inserts in the λgt11 vector. 1 µl of phage lysate (containing 50-100 ng DNA), prepared

Table 1. *DNA Primers Used In the Present Study.*

Primer	Sequence (5′ to 3′)
T3	ATTAACCCTCACTAAAG
T7	AATACGACTCACTATAG
λgt11 (forward)	GGTGGCGACGACTCCTGGAGCCCG
λgt11 (reverse)	TTGACACCAGACCAACTGGTAATG

by re-suspending a single isolated plaque in 100 µl of suspending medium (SM, containing 100 mM NaCl, 0.2% Mg $SO_4 \cdot 7H_2O$, 50 mM Tris-Cl pH 7.5 and 0.01% gelatin), was used as a template in a 50 µl reaction volume. The reaction mixture included 50 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.0025% β-mercaptoethanol, 0.01% BSA, 0.2 mM of each of dATP, dCTP, dGTP, dTTP, 0.12 mM of each λgt11 primer, and 2.5 Units of Taq polymerase. The reaction solution was covered with a drop of mineral oil to prevent evaporation during the reaction cycles.

PCR was carried out in an AutoGene II Thermal Cycler (Grant Instruments Cambridge Ltd.). The reaction mixture was initially heated to 94 °C for 7 min to disintegrate phage protein coats and release DNA, followed by 30 cycles of 1 min at 60 °C, 1 min at 72 °C and 1 min at 92 °C. A final cycle of 1 min at 60 °C and 4 min at 72 °C was completed before cooling to 23 °C . PCR products were fractionated on 1.2 % agarose gels, and DNA bands of interest were excised and gene-cleaned using NaI and glass milk (Vogelstein and Gillespie, 1979).

2.14. Southern Blot Analyses

Pea genomic DNA (10 µg) was digested with the appropriate restriction endonuclease and fractionated on a 0.8% agarose gel according to Ausubel et al. (1992). After partial depurination in 0.2 M HCl, denaturation in 0.5 M NaOH/1.5 M NaCl and neutralization in 3 M sodium acetate, the gel was capillary-blotted to a Biotrans$^+$ nylon membrane (ICN Biomedical, Inc.) following the procedure of Southern (1979).

Restriction fragments or PCR products were radiolabeled (see Section 2.12) and used to probe for complementary DNA bands that were transferred onto the nylon membranes (Ausubel et al., 1992). Prehybridization, hybridization and washing were performed according to the ICN instruction manual. Kodak XAR films were used for autoradiography and developed on a Kodak M35A X-OMAT Processor.

2.15. Northern Blot Analyses

For the electrophoresis of RNA, the buffer tank, gel casting chamber and combs were all sequentially washed with detergent, dried with ethanol, soaked for 10 min in 3% H_2O_2 (v/v) and then rinsed thoroughly with DEPC-treated H_2O (see Section 2.10). All glassware was baked at 180 °C overnight before use (Wilkinson, 1991). 10-20 μg of total RNA was fractionated on formaldehyde agarose gels and the Promega RNA markers were included to allow size estimates. RNA was then transferred by capillary action from gels to Bio-Rad Zeta-Probe Blotting membranes. The gel electrophoresis procedures, RNA transfer, membrane prehybridization and hybridization protocols of Ausubel et al. (1992) were followed without modification. ^{32}P-labeled restriction fragments were used as probes to detect mRNA(s) of interest. Kodak XAR films were used for autoradiography with a intensifying screen. The exposure time was up to two weeks.

2.16. Isolation of cDNA Encoding Cytosolic DHY-CYC

Immunoscreening of a λgt11 Expression Library

A λgt11 expression library, constructed using leaves of 10 d light-grown pea seedlings was screened (more than $5x10^5$ pfu) using polyclonal DHY-CYC-Ab. *E. coli* strain Y1090r⁻ was used as host cells. Amersham Hybond™ C-extra nitrocellulose filters and ECL™ were employed for plaque lifts and immuno-detections. The protocols of Sambrook et al. (1989), and those outlined in the Amersham Tech Tip 133 were followed. A positive plaque was detected and picked up as a plug using the small end of a autoclaved Pasteur pipette. Phages were lysed from the agar plug in 100 μl of suspending medium containing a drop of chloroform.

λDNA, contained in the positive plaque, was prepared from plate lysates by DEAE-cellulose column chromatography and phenol/chloroform/isoamylalcohol extraction (Ausubel et al., 1992). λDNA (2 μg) was further purified by fractionation on a 0.8% agarose gel followed by gene-cleaning (Vogelstein and Gillespie, 1979). The purified λDNA was subsequently subjected to Eco RI digestion and ligation to phagemids pBluecript SK (+/-) (Promega) according to the supplier's instruction manual. The phagemids were then transformed into ampicillin sensitive competent *E. coli* cells (strain XL-1 Blue) and grown on agar plates containing 100 μg/ml ampicillin. 16 isolated colonies were recovered and amplified. Phagemid DNA was isolated by the alkaline lysis mini-prep method of Sambrook et al. (1989), and subsequently fractionated on a 1% agarose gel after Eco RI digestion. A phagemid containing the appropriate insert (designated Dλ2) was sequenced in both directions using T3 and T7 primers.

Screening of a λgt11 Library Using Radiolabeled DNA Fragments as Probes

The DNA clone (Dλ2) obtained from immunoscreening contained a sequence that was truncated at 5′ end as judged by a) the amino acid sequence translated on the PCGENE program; and b) comparison with the published sequences of other species. Dλ2 was therefore used as a probe for further screening. Amersham Hybond-N$^+$ nylon filters were utilized for plaque lifts and *E. coli* strain Y1090r$^-$ acted as host cells. More than 5x10^5 pfu from a 10 d light-grown pea λgt11 library were screened using the procedures outlined in the λZAP Product Protocol of Promega. Filters were probed with ^{32}P-labeled Dλ2 obtained by EcoR I digestion of mini-prep prepared phagemids (see Section 2.12). Eighteen positive plaques were recovered and 8 of these were subjected to a secondary screening. This resulted in 7 separate, positive plaques. Inserts in these 7 clones were amplified by PCR using λgt11 primers and fractionated on a 1.2% agarose gel for size estimates. Only 1 (DD7) of the 7 clones contained an insert with a size larger than Dλ2. The DNA band of DD7 was excised and gene-cleaned for sequencing in both directions using λgt11 primers.

Although DD7 was about 150 nucleotides longer than Dλ2 at both the 5′ and 3′ ends, it however was still incomplete at the 5′ end as judged by the translated amino acid sequence. Restriction enzyme cut sites on DD7 were therefore scanned by PCGENE and a single Hind III digestion site was found which resulted in a 377 bp fragment from the 5′ end. Later, this 377 bp DNA fragment (designated 337bp) was used in the secondary screening of the remaining 10 positive plaques obtained from the primary screening. Three separate positive plaques were obtained and the sequencing results indicated that none of them contained a DNA sequence that was longer than DD7. A λgt11 library, constructed using leaves of 9 d dark-grown pea seedlings, was therefore screened using the 377bp DNA fragment as a probe. 6.5×10^5 pfu were screened using this probe. After primary and secondary screenings, 7 positive plaques were recovered. Three of them were found to be identical and contained a nucleotide sequence encoding the full-length amino acid sequence of cytosolic DHY-CYC.

3. RESULTS

3.1. The Occurrence of SYN, DHY and CYC Activities in Pea Leaf Extracts

SYN, DHY and CYC activities were readily detected in pea leaf extracts when leaves were homogenized in Buffer A (Table 2). There is good evidence that, in plant leaves, folate derivatives are present not only in the cytoplasm, but also in the mitochondrial and chloroplast compartments (Cossins, 1984 and 1987). It is therefore conceivable that these three folate-dependent enzymes may also occur in these organelles.

Pea leaf mitochondria (Douce et al., 1987) and chloroplasts (Schuler and Zielinski, 1989) were isolated using Percoll gradients. Prior to preparing enzyme extracts of the isolated organelles, Triton X-100, the widely used detergent in organelle lysis, was tested for its effect on SYN, DHY and CYC activities in pea leaf extracts. The Triton X-100 treatment at 0.1% (v/v) appeared to have no significant effect on these enzyme activities (Table 2), indicating that in pea leaves, the bulk of these proteins are probably not associated with membranes or membrane-enclosed organelles.

During the preparation of mitochondrial enzyme extracts, it was observed that besides the Triton X-100 treatment, sonication appeared to enhance the recovery of each enzyme activity (Table 3). No enzyme activities were detected when only Buffer A was used. It is noteworthy that the specific activities of SYN, DHY and CYC in pea leaf mitochondria (Table 3) were similar to those found in whole-leaf extracts (Table 2). Table 3 also indicates that, compared to Triton X-100, Na deoxycholate was a poorer detergent in the recovery of these activities from pea leaf mitochondrial extracts. The same

Table 2. *The Effect of Triton X-100 on the DHY, CYC and SYN Activities of Whole-Leaf Extracts of 14 d Pea Seedlings.*

10 g of pea leaves were homogenized in 20 ml buffer A (see Section 2.5) with or without Triton X-100. Crude extracts were centrifuged at 8,000 x g for 20 min, and the supernatants were assayed for each enzyme activity. Activities are expressed in nmol min^{-1} mg^{-1} protein and are the averages of three separate determinations (± standard errors).

Enzymes	Buffer A	Buffer A + 0.1% Triton X-100	Buffer A + 0.5% Triton X-100
Dehydrogenase	5.45±0.14	5.70±0.15	3.50±0.10
Cyclohydrolase	2.73±0.09	2.54±0.11	1.63±0.05
Synthetase	9.15±0.21	10.83±0.26	8.55±0.22

Table 3. *The Effect of Sonication and Detergent Treatments on the Specific Activities of SYN, DHY and CYC in Pea Leaf Mitochondrial Extracts.*

Approximately 60 mg of mitochondrial protein were used in each treatment. The composition of Buffer A and the sonication procedures are described in Sections 2.4 and 2.5 respectively. Data are the averages of three separate determinations (± standard errors)

Treatments	Synthetase	Dehydrogenase (nmol min^{-1} mg^{-1})	Cyclohydrolase
Buffer A only	n.d.[a]	n.d.[a]	n.d.[a]
Buffer A + sonication	0.34 ± 0.01	n.d.[a]	n.d.[a]
Buffer A + 0.1% Triton X-100	2.15 ± 0.07	1.70 ± 0.03	0.90 ± 0.02
Buffer A + sonication + 0.1% Triton X-100	5.30 ± 0.12	3.21 ± 0.07	2.26 ± 0.07
Buffer A + sonication + 0.1% Na deoxycholate	n.d.[a]	0.25 ± 0.01	0.38 ± 0.01

[a], not detected.

sonication and 0.1% (v/v) Triton X-100 treatments were used in the preparation of enzyme extracts of isolated chloroplasts. However, none of the three activities could be detected by the standard assays in fractions of thylakoid membrane, stroma and whole chloroplasts (data not shown).

The cellular distribution of SYN, DHY and CYC activities in pea leaf therefore appeared to be principally between the cytosolic and mitochondrial compartments. It is clear from work of Douce et al. (1987) that PVP is an important component of isotonic mitochondrial isolation buffers because it removes phenolic compounds. These latter compounds are known to cause the rapid inactivation of mitochondrial activity. The effect of PVP on SYN, DHY and CYC activities in whole-leaf extracts was therefore examined when isotonic extracts were centrifuged to recover cytosolic and mitochondrial fractions (Table 4). Of the three types of PVP tested, soluble PVP-25 displayed the best incremental effect on enzyme activity and was therefore used in subsequent work. When the distribution of SYN, DHY and CYC activities was examined in cytosolic and mitochondrial fractions, it was clear that the bulk of these activities were cytosolic. Based on the total enzyme units recovered in these assays, less than 1% of each activity was present in the mitochondria (Table 5).

3.2. The Association of Cytosolic DHY and CYC Activities in Pea Leaves

3.2.1. Co-purification of cytosolic DHY and CYC activities

Table 4. *The Effect of PVP on DHY, CYC and SYN Activities in Whole Leaf Extracts of 14 d Pea Seedlings.*

10 g of pea seedlings were homogenized in 20 ml of buffer A (see Section 2.5) with or without 0.5 % (w/v) PVP of different molecular weights. Crude leaf extracts were centrifuged at 8,000 x g for 20 min, and the supernatants were assayed for each activity. Enzyme activities are expressed in nmol min^{-1} mg^{-1} protein. Data are the averages of three separate determinations.

Enzymes	Buffer A	Buffer A + PVP-40	Buffer A + PVP-25	Buffer A + HMW PVP[a]
Dehydrogenase	4.46±0.15	5.40±0.14	8.44±0.28	6.88±0.22
Cyclohydrolase	2.17±0.08	2.41±0.10	2.12±0.09	1.82±0.07
Synthetase	5.98±0.16	7.07±0.22	9.46±0.31	n.d.[b]

[a], insoluble high molecular weight PVP; [b], not detected.

Table 5. *Distribution of SYN, DHY and CYC in Cytosolic and Mitochondrial Extracts.*

Enzyme Activity	Cytosolic (12,000x g supernatant)	Mitochondrial Pellet Sonicated + Triton X-100
10-HCO-H_4PteGlu synthetase		
Total units[a]	62.5 (10.5)	0.32 (5.32)
% of cytosolic activity	100	0.52
5,10-CH_2-H_4PteGlu dehydrogenase		
Total units[a]	45.4 (7.63)	0.20 (3.24)
% of cytosolic activity	100	0.44
5,10-CH^+-H_4PteGlu cyclohydrolase		
Total units[a]	25.8 (4.33)	0.14 (2.21)
% of cytosolic activity	100	0.54
Alcohol dehydrogenase		
Total units[a]	41.3	n.d.[b]
% of cytosolic activity	100	n.d.[b]
Succinate dehydrogenase		
Total units[a]	<0.01	1.01
% of cytosolic activity	n.a[c]	n.a.[c]

[a], expressed in μmol min^{-1}. [b], not detected. [c], not applicable. Values in brackets are specific enzyme activities (nmol min^{-1} mg^{-1} protein).

To purify the DHY and CYC activities of whole-leaf extracts, protein was fractionated by $(NH_4)_2SO_4$ precipitation and column chromatography on heparin agarose, Matrex Green A, Sephadex G-75 and Matrex Orange A as summarized in Table 6. To minimize proteolysis during purification, PMSF was included in all of the buffers used in this protocol. The ratio of DHY to CYC activities remained relatively constant during this fractionation, indicating that both activities co-purified through steps 1-7 of the protocol. At Step 5 of the protocol, the bulk of SYN activity in these extracts was effectively removed (Figure 5). By Step 7, SYN activity was not detected and the purifications achieved were about 3,900-fold for DHY activity and 4,400-fold for CYC. Recovery of these activities was 8-9%. Considering the very low levels of both activities in whole mitochondrial extracts (Table 5), it follows that Step 7 protein was mainly derived from the cytosolic compartment. SDS-PAGE of protein from Step 7 revealed a single, silver-stained protein band with an average M_r of 38,000 (Figure 6). Sephadex G-75 chromatography in the presence of molecular weight marker proteins showed that the DHY-CYC fractions had an apparent molecular weight of about 58 kDa (Figure 7). A value of 60 kDa was obtained when Step 7 protein was chromatographed on a calibrated column of Sephacryl S-200 (Figure 8). Based on these data it appears likely that the DHY-CYC protein is homodimeric.

3.2.2. Immunoblots using antibodies raised against purified cytosolic DHY-CYC protein

Table 6. *Copurification of DHY and CYC Activities During Fractionation of Pea Whole-leaf Extracts.*

Fractionation Step	Protein (mg)	DHY Activity Total[a]	DHY Activity Specific[b]	CYC Activity Total[a]	CYC Activity Specific[b]	Ratio (DHY/CYC)
1.Crude Homogenate	1880	16.90	0.01	6.34	0.003	2.66
2.Streptomycin SO_4	1143	16.40	0.01	6.05	0.005	2.71
3.55-75% $(NH_4)_2SO_4$	246	6.94	0.03	2.85	0.012	2.43
4.Heparin Agarose	37.5	9.94	0.26	3.32	0.090	2.99
5.Matrex Green A	6.0	5.76	0.96	2.41	0.402	2.39
6.Sephadex G-75	2.5	4.95	1.96	2.09	0.840	2.37
7.Matrex Orange A	0.038	1.32	34.7	0.57	15.00	2.33

[a], μmol min^{-1}. [b], μmol min^{-1} mg^{-1}.

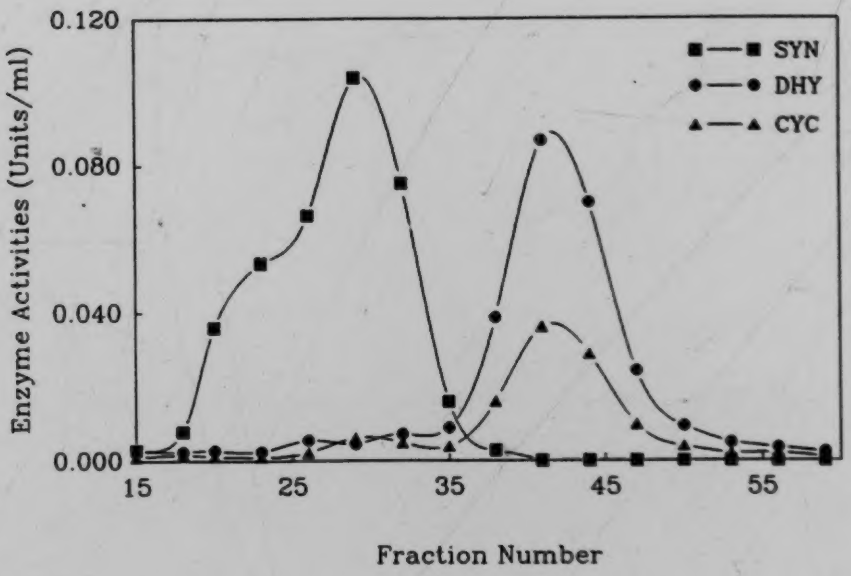


Figure 5. *Separation of DHY and CYC from SYN Activities.*

After heparin agarose chromatography Step 4 protein (see Table 6) was applied to a 5 x 10 cm column of Matrex Green A (see Section 2.5). Fractions of 3 ml were collected and assayed for SYN, DHY and CYC activity. After loading the enzyme extract, the column was washed with 30 ml of 0.45 M NaCl in Buffer A (from fractions 25 to 34), followed by an elution with a linear NaCl gradient from 0.45 to 1.0 M (see Section 2.5).

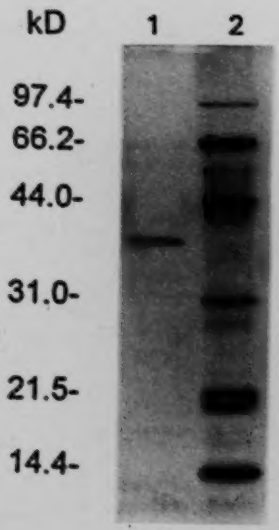

kD
1
2
97.4-
66.2-
44.0-
31.0-
21.5-
14.4-

Figure 6. *SDS-PAGE of Cytosolic DHY-CYC Protein.*

DHY-CYC protein in combined fractions after Matrex Orange A purification. DHY-CYC protein (lane 1), molecular weight standards (lane 2).

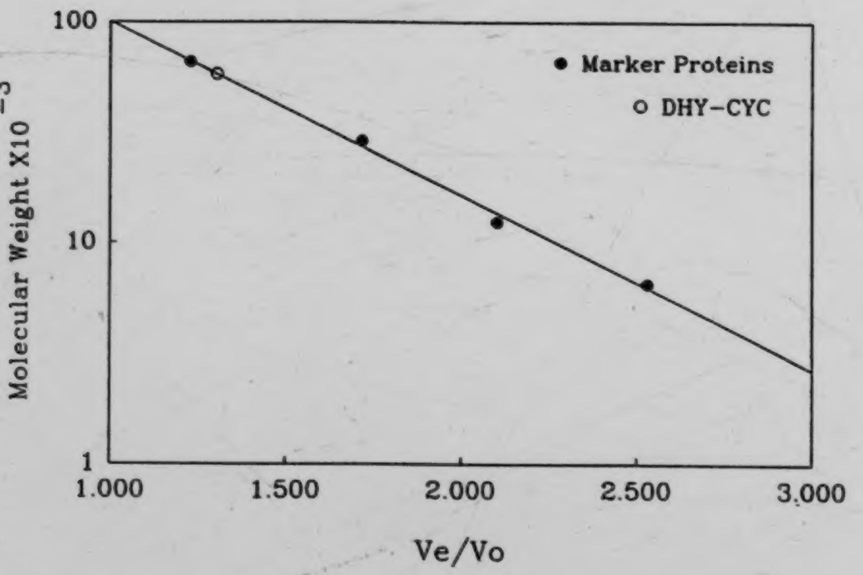


Figure 7. *Determination of the Native Molecular Weight of Cytosolic DHY-CYC Protein by Gel Filtration Chromatography on Sephadex G-75.*

Partially purified enzyme (Step 5 protein, see Table 6) was applied to a 2.5 x 80 cm column of Sephadex G-75 (see Section 2.5). Protein standards included BSA (66 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa), and aprotinin (6.5 kDa). Blue Dextran (2,000 kDa) was employed to determine V_o. The M_r value (58 kDa)of the DHY-CYC protein was the average of three separate determinations.

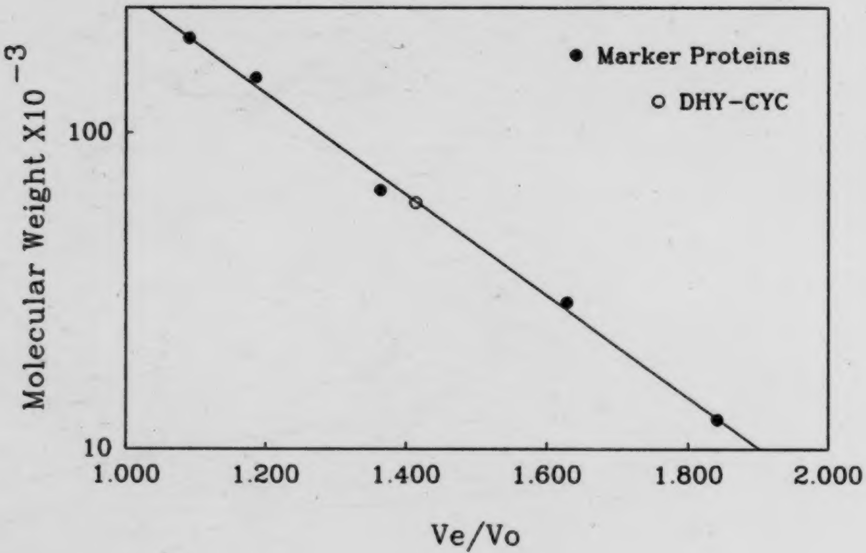


Figure 8. *Determination of the Native Molecular Weight of Cytosolic DHY-CYC Protein by Gel Filtration Chromatography on Sephacryl S-200.*

Purified DHY-CYC protein (Step 7 protein in Table 6) was applied to a 2.5 x 80 cm column of Sephacryl S-200. Gel filtration chromatography was carried out as described in Section 2.5. Protein standards were cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), BSA (66 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa). Blue Dextran (2,000 kDa) was employed to determine V_o. The M_r value (60 kDa) of the DHY-CYC protein was the average of three separate determinations.

Polyclonal antibodies were raised in rabbits against step 7 DHY-CYC cytosolic protein (Table 6). The titer of the resulting polyclonals was tested by ELISA as shown in Figure 9. Following these assays, IgG was partially purified and lyophilized (see Section 2.7) prior to being used in immunological studies. It is clear from Figure 10 that DHY-CYC-Ab cross-reacted with a single protein band (M_r of approximately 38,000) in whole-leaf and mitochondrial extracts. The mobility of these bands was like that observed when the purified cytosolic DHY-CYC was revealed by silver-staining of SDS-PAGE gels (Figure 6). However, when chloroplast extracts were subjected to immunoblot analyses, no DHY-CYC-Ab cross-reacting bands were detected (Figure 11).

3.3. The Mono-functional Nature of Cytosolic SYN

3.3.1. Purification of cytosolic SYN

The purification of mono-functional SYN proteins has been reported for spinach leaves (Nour and Rabinowitz, 1991) and pea cotyledons (Kirk et al., 1994). In the present work an improved protocol was employed that gave homogeneous SYN protein after chromatography on DEAE-52 cellulose, heparin agarose, and hydroxyapatite (Table 7). By Step 5, SYN was purified 650-fold with a recovery of about 17%. The level of activity recovered, compared to that found in whole mitochondrial extracts (Table 5), suggests that Step 5 protein was mainly derived from the cytosolic form of this enzyme. SDS-PAGE revealed a single, silver-stained protein band with an average M_r of 66,000 (Figure 12). This protein lacked DHY and CYC activities and gel filtration on Sephacryl S-300 in

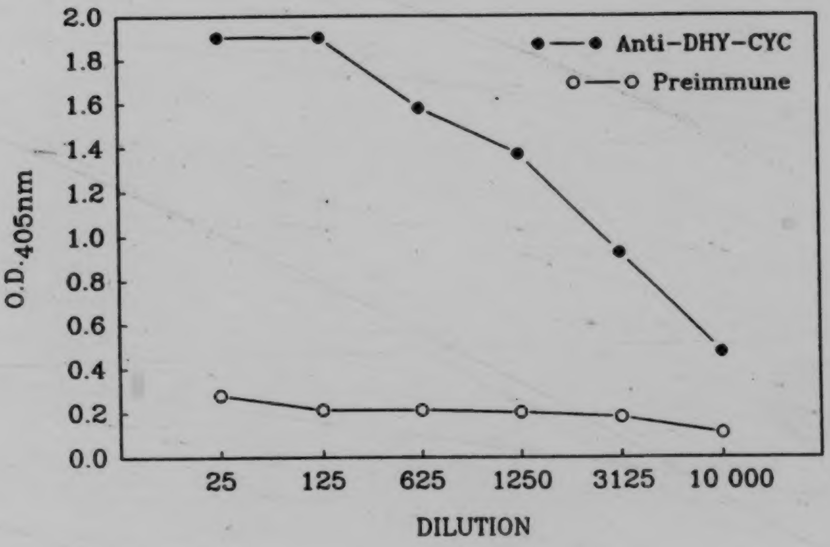


Figure 9. *Reactivity of Antisera Against DHY-CYC Protein.*

ELISA measurements (see Section 2.7) employed microtiter wells coated with purified DHY-CYC protein and containing dilutions of anti- or pre-immune sera. The secondary antibody was goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma). Each value is the mean of triplicate determinations. The standard error was less than 5%.

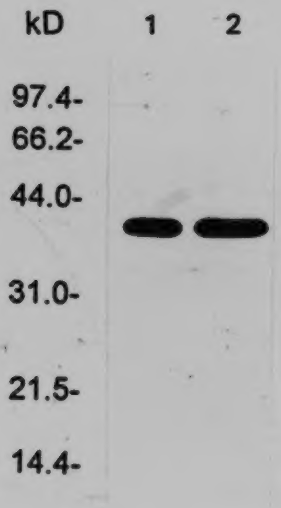


Figure 10. *Immunoblots of Whole-leaf and Mitochondrial Extracts Using DHY-CYC-Ab.*

Whole-leaf extract, 20 µg protein (lane 1); extract of gradient-purified mitochondria, 35 µg protein (lane 2). Extracts were subjected to SDS-PAGE and Western blot analyses as described in Section 2.9.

Figure 11. *SDS-PAGE (A) and Immunoblot (B) of Chloroplast, Mitochondrial and Whole-leaf Extracts Using DHY-CYC-Ab.*

Enzyme extracts were prepared as described in Section 2.4. For SDS-PAGE, protein bands were detected by Ag staining. Gradient-purified chloroplasts, 35, 28, 21, 14 and 7 µg protein (lanes 1 to 5 respectively); molecular weight standards (lane 6); thylakoid membrane extract, 20 µg protein (lane 7); chloroplast stromal extract, 30 µg protein (lane 8); gradient-purified mitochondria, 18 µg protein (lane 9); whole leaf extract, 20 µg protein (lane 10). The approximate positions of marker proteins are indicated by horizonal lines (B, lane 6).

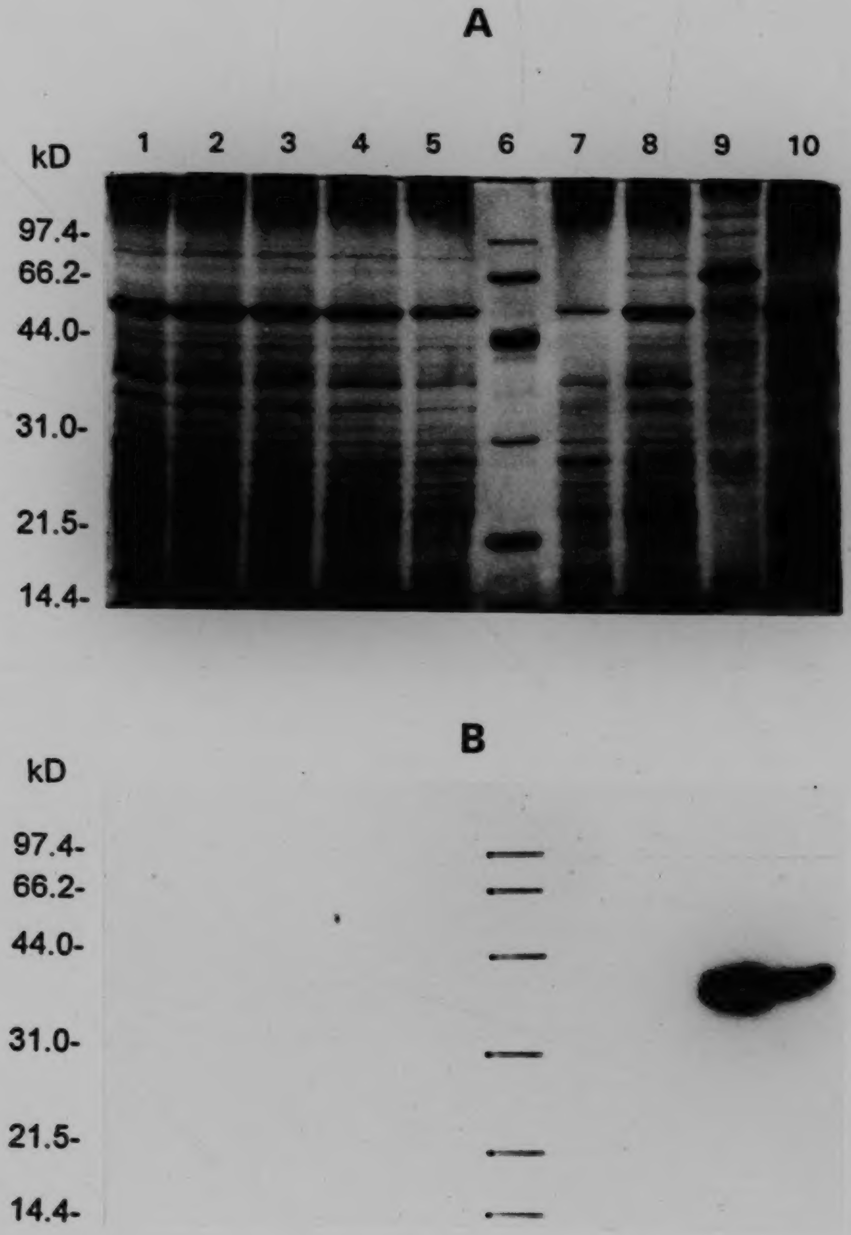

A
kD
1
2
3
4
5
6
7
8
9
10
97.4-
66.2-
44.0-
31.0-
21.5-
14.4-
B
kD
97.4-
66.2-
44.0-
31.0-
21.5-
14.4-

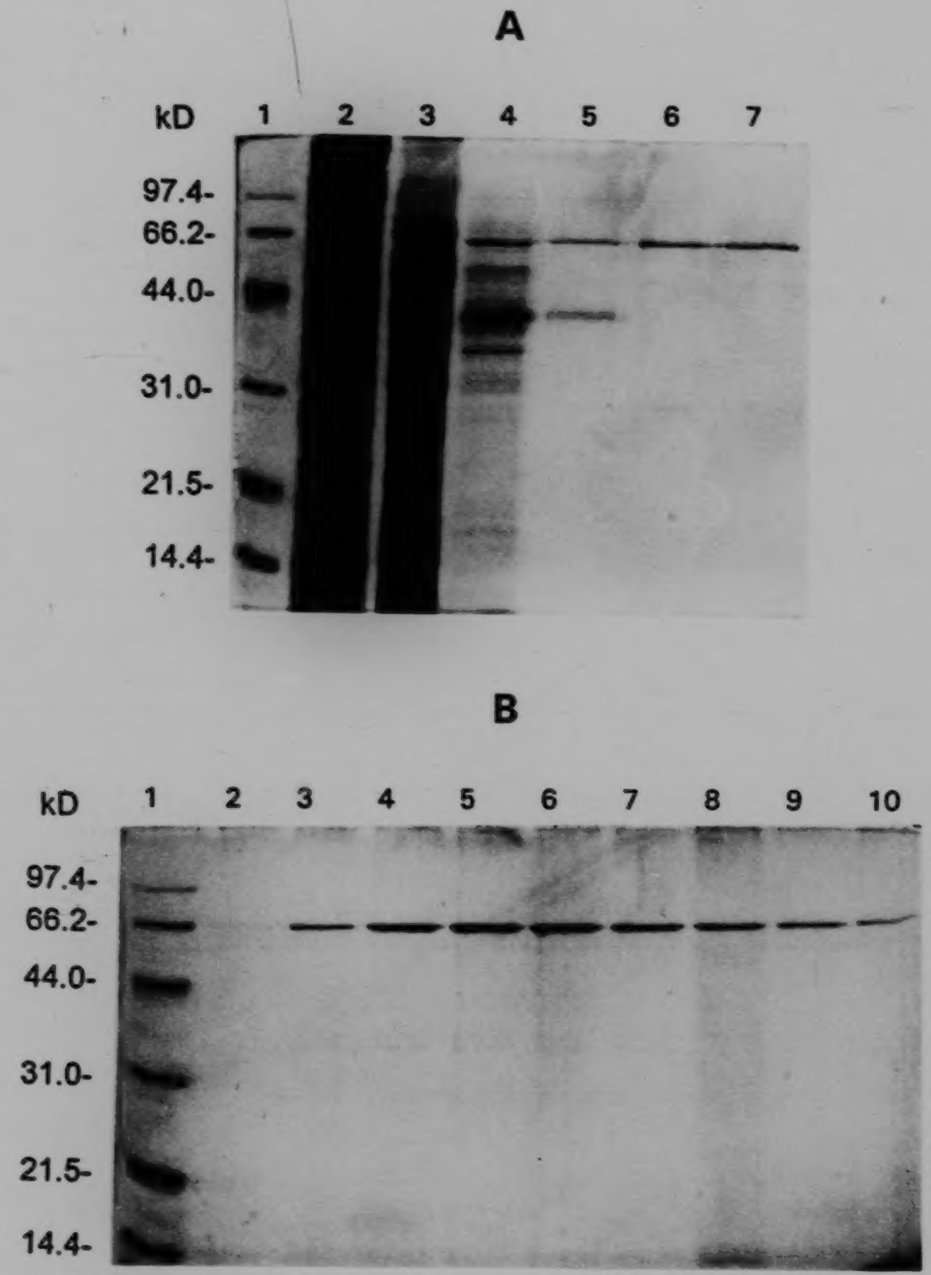
Table 7. *Purification of SYN Activity from Pea Whole-leaf Extracts.*

Fractionation Step	Protein (mg)	Activity Total[a]	Activity Specific[b]	Purification (fold)	Recovery (%)
1. Initial extract	2259	12.29	0.005	1	100
2. 50-70% $(NH_4)_2SO_4$	722	7.03	0.010	2	57.2
3. DEAE-52 cellulose	8.04	5.34	0.664	123	43.4
4. Heparin agarose	1.45	3.12	2.155	400	25.4
5. Hydroxyapatite	0.58	2.04	3.565	650	16.6

[a], Expressed in μmol min^{-1}. [b], Expressed in μmol min^{-1} mg^{-1}.

Figure 12. *SDS-PAGE of Cytosolic SYN Protein.*

Protein bands were detected by Ag staining. A, proteins from combined enzyme-active fractions during different SYN fractionation steps. Molecular weight standards (lane 1); whole leaf extract (lane 2); 50-70% $(NH_4)_2SO_4$ fraction (lane 3); after DEAE-52 cellulose column (lane 4); after heparin agarose column (lane 5); after hydroxyapatite column (lane 6 and 7). B, SYN protein in enzyme-active fractions recovered from the hydroxyapatite column. Molecular weight standards (lane 1); fractions 5 to 13 (lanes 2 to 10 respectively). The specific enzyme activities (nmol min^{-1} ml^{-1}) of fractions 5 to 13 were: 4.8, 21, 62, 93, 86, 48, 34, 20, and 13 respectively.


A
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4
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97.4-
66.2-
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31.0-
21.5-
14.4-
B
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66.2-
44.0-
31.0-
21.5-
14.4-

the presence of molecular weight marker proteins indicated an average M_r value of 130,000 (Figure 13). Thus the SYN of pea leaves, like that of spinach leaves and pea cotyledons, appears to be a mono-functional protein that is homodimeric.

3.3.2. Immunoblots using antibodies raised against purified cytosolic SYN protein

Polyclonal antibodies were raised in rabbits against step 5 SYN cytosolic protein (Table 7). The titer of these polyclonals was determined by ELISA as shown in Figure 14. Before use in further immunological studies, IgG was partially purified and lyophilized (see Section 2.7). Immunoblots using the resulting purified SYN-Ab as primary antibody (Figure 15B) revealed two cross-reacting bands (44 kDa and 40 kDa) in mitochondrial extracts, one band (66 kDa) in purified SYN and four major cross-reacting bands (63 kDa; 45.5 kDa; 44 kDa and 28 kDa) in whole-leaf extracts. However, no SYN-Ab cross-reacting band was detected in chloroplast extracts when they were examined in immunoblot analyses (Figure 16).

3.4. The Occurrence of Bifunctional DHY-CYC and Mono-functional SYN in Other Plant Species

Leaf extracts of pea, bean, wheat, corn and barley were fractionated with $(NH_4)_2SO_4$, heparin agarose and Matrex Green A following the protocol for purification of pea leaf DHY-CYC (see Section 2.5). During the latter step, the bulk of the applied SYN activity was not retained by the affinity column (Table 8). In contrast, DHY and CYC activities appeared to be co-eluted in response to the applied chloride gradient.

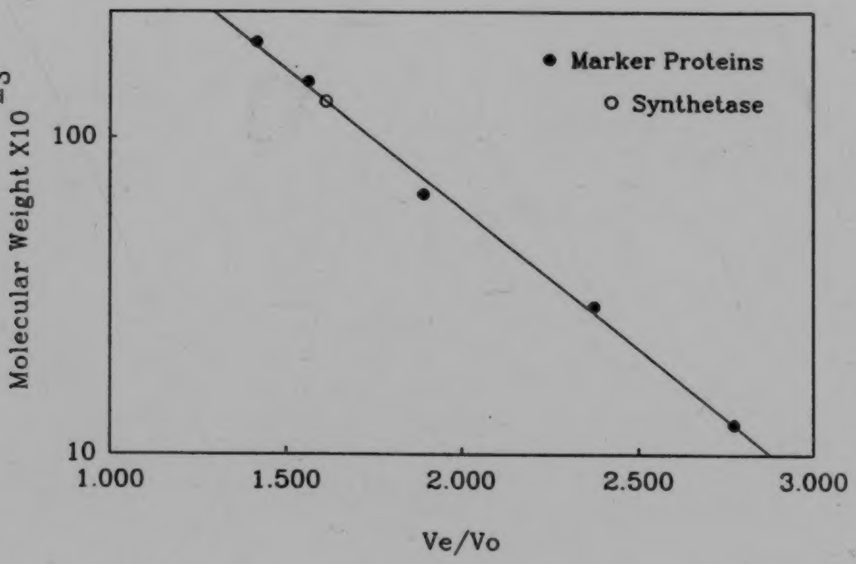


Figure 13. *Determination of Native Molecular Weight of Cytosolic SYN Protein.*

Partially purified enzyme solution (Step 4 protein) was applied to a 3 x 80 cm column of Sephacryl S-300 (see Section 2.6). Protein standards were cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), BSA (66 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa). Blue Dextran (2,000 kDa) was employed to determine Vo. The M_r value (130 kDa) of the SYN protein was the average of three separate determinations.

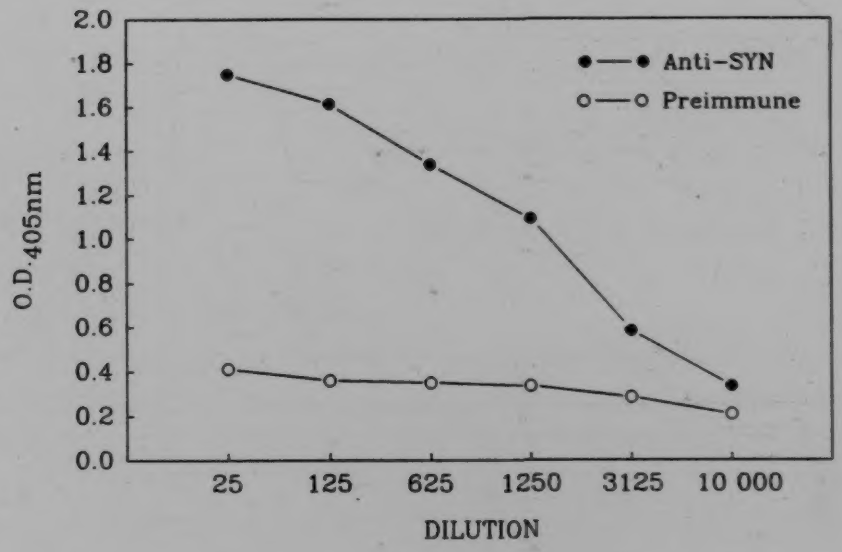
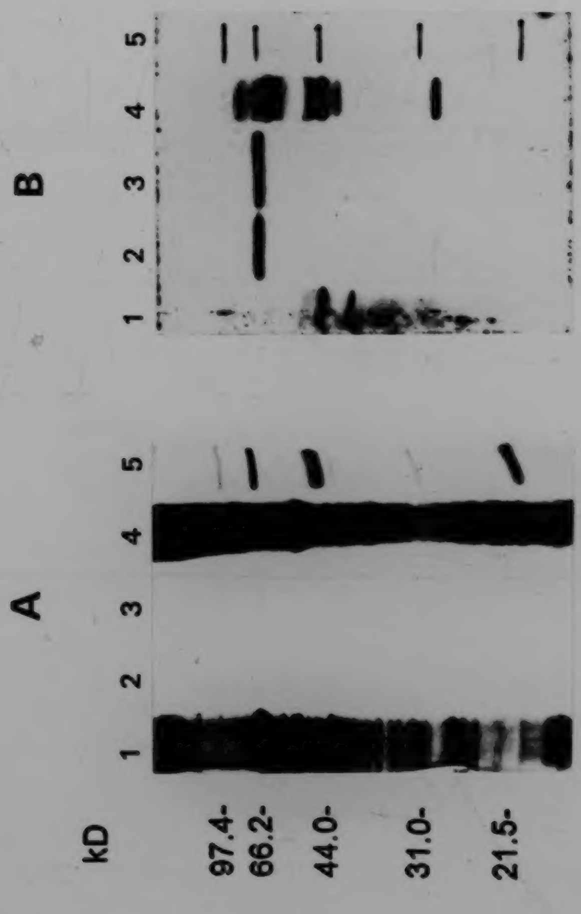


Figure 14. *Reactivity of Antisera Against SYN Proteins.*

ELISA measurements (see Section 2.7) employed microtiter wells coated with purified SYN protein and containing dilutions of anti- or pre-immune sera. The secondary antibody was goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma). Each value is the mean of triplicate determinations. The standard error was less than 5%.

Figure 15. *SDS-PAGE (A) and Immunoblots (B) of Whole-leaf and Mitochondrial Extracts Using SYN-Ab.*

Gradient-purified mitochondria, 30 µg protein (lane 1); purified cytosolic SYN, 40 ng and 50 ng protein (lanes 2 and 3 respectively); whole-leaf extract, 40 µg protein (lane 4).


A
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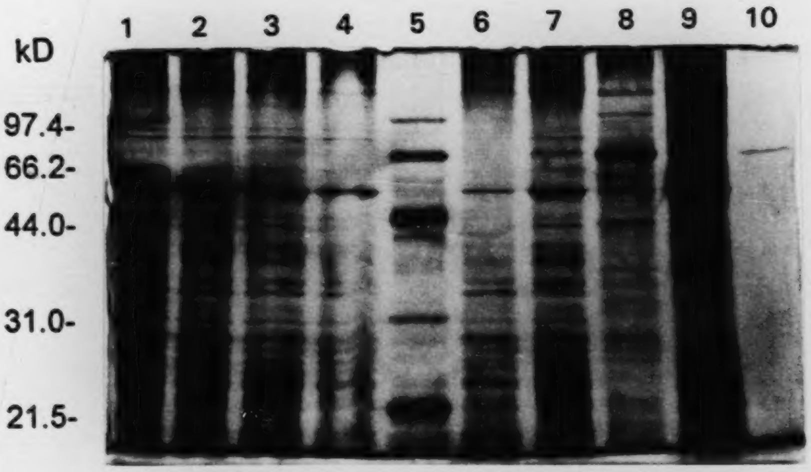
kD
97.4-
66.2-
44.0-
31.0-
21.5-

Figure 16. *SDS-PAGE (A) and Immunoblot (B) of Chloroplast, Mitochondrial and Whole-leaf Extracts Using SYN-Ab.*

Enzyme extracts were prepared as described in Section 2.4. For SDS-PAGE, protein bands were detected by Ag staining. Gradient-purified chloroplasts, 35, 28, 21, and 7 µg protein (lanes 1 to 4 respectively); molecular weight standards (lane 5); thylakoid membrane extract, 20 µg protein (lane 6); chloroplast stromal extract, 30 µg protein (lane 7); gradient-purified mitochondria, 18 µg protein (lane 8); whole leaf extract, 35 µg protein (lane 9); purified cytosolic SYN, 40 ng protein (lane 10). The approximate positions of marker proteins are indicated by horizonal lines (B, lane 5).

A


1
2
3
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10
kD
97.4-
66.2-
44.0-
31.0-
21.5-

B

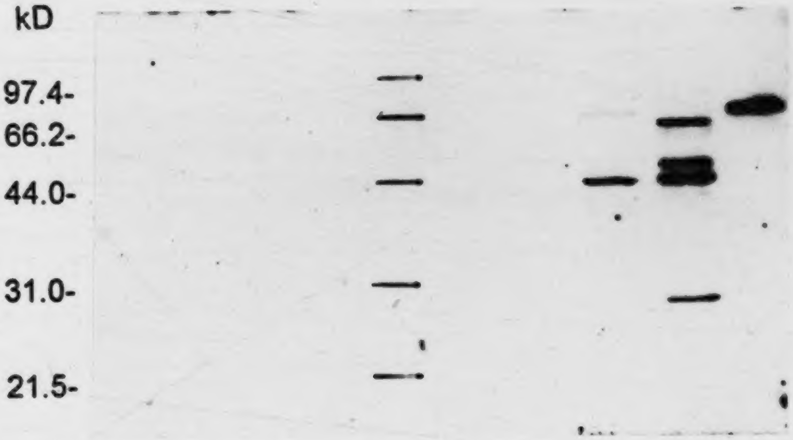

kD
97.4-
66.2-
44.0-
31.0-
21.5-

Table 8. *Separation of SYN from DHY and CYC Activities in Plant Leaf Extracts.*

Species	Enzyme	Applied Activity to column (μmol min^{-1})	Recovered from Matrex Green A (%)	
			Wash Buffer	Elution Buffer
Pea	Synthetase	5.99	60	n.d.[a]
	Dehydrogenase	9.90	n.d.[a]	58
	Cyclohydrolase	3.30	n.d.[a]	51
Bean	Synthetase	1.08	72	n.d.[a]
	Dehydrogenase	0.60	n.d.[a]	59
	Cyclohydrolase	0.18	n.d.[a]	54
Wheat	Synthetase	4.98	68	n.d.[a]
	Dehydrogenase	3.30	14	53
	Cyclohydrolase	3.72	13	51
Corn	Synthetase	0.72	65	n.d.[a]
	Dehydrogenase	0.132	15	66
	Cyclohydrolase	0.60	12	64
Barley	Synthetase	0.90	55	n.d.[a]
	Dehydrogenase	1.98	17	55
	Cyclohydrolase	1.08	18	58

[a], not detected.

These fractions were pooled and concentrated by ultrafiltration prior to electrophoresis and immunological studies. DHY-CYC-Ab were used to examine the DHY- and CYC-active fractions for proteins that might be structurally related to pea DHY-CYC. The results of indirect, competitive ELISA assays of these extracts are summarized in Table 9. As expected, significant cross-reactivity occurred with samples of purified, homogeneous DHY-CYC protein from pea leaves. The antibodies also cross-reacted with the DHY and CYC containing fractions of four other plants and with the $(NH_4)_2SO_4$-fractionated protein of two *Neurospora* strains that are rich in C_1-THF synthase activity.

In further studies, SYN-Ab and DHY-CYC-Ab were used in immunoblot analyses to examine extracts of other species of higher plants, fungi and bacteria for cross-reacting proteins (Tables 10 and 11). In the higher plant species, a single DHY-CYC-Ab cross-reacting protein band with an M_r value of approximately 40,000 was detected (Table 11). With the exception of bean, SYN-Ab detected more than one cross-reacting protein bands in the higher plant extracts (Table 10). In each of these higher plants, the apparent M_r of the DHY-CYC-Ab cross-reacting protein bands were distinct from the M_r values of proteins which cross-reacted with SYN-Ab. It therefore appears likely that these plants express SYN and DHY/CYC proteins that are not structurally associated (Tables 10 and 11). Both SYN-Ab and DHY-CYC-Ab cross-reacted with the subunit of yeast tri-functional C_1-synthase (M_r=110,000). A protein band (M_r of approximately 38,000) was also revealed when extracts of *Neurospora*, mushroom (*Agaricus brunnescens*) and *Euglena* were probed with DHY-CYC-Ab. Interestingly, in species other than higher

Table 9. *Cross Reactivity of DHY-CYC-Ab in ELISA.*

Indirect competitive ELISA assays of plant and *Neurospora* extracts. ELISA assays were conducted as described in the Materials and Methods. Higher plant leaf extracts were fractionated (see Table 6) and concentrated by ultrafiltration. *Neurospora* mycelial protein (50-65% saturation with $(NH_4)_2SO_4$) was desalted prior to assay. (Data are absorbance readings at 405 nm).

Extract	Corn	Pea	Wheat	Bean	Barley	*Neurospora*	
Dilution						Wild Type	Formate Mutant
1:5	0.31	0.43	0.25	0.38	0.29	0.14	0.13
1:50	0.65	0.62	0.46	0.65	0.58	0.23	0.21
1:200	0.64	0.64	0.59	0.71	0.71	0.43	0.41
1:1000	0.75	0.69	0.65	0.75	0.84	0.82	0.71
1:3000	0.74	0.79	0.74	0.82	0.79	1.00[a]	0.94[a]
Controls	0.76	0.75	0.78	0.80	0.81	1.08	1.03

[a], 1:5000 dilution of *Neurospora* extract.

Table 10. *Immunoblot Survey of Species for Immunologically Related Proteins Using SYN-Ab.*

Crude enzyme extracts were prepared by homogenizing tissues (leaves for higher plants) in Buffer B (see Section 2.6) followed by centrifugation at 8,000 x g for 20 min. Supernatants were saved and used for enzyme assays and SDS-PAGE. Samples for SDS electrophoresis were prepared by following the standard protocol (see Section 2.9). The relative intensity of cross-reacting protein bands is indicated as dark (bold), median (normal), and light (italic).

SDS-treated extracts	SA[a] of Extracts (nmol min^{-1} mg^{-1})	Enzyme Applied (nmol min^{-1} lane^{-1})	Cross-reacting Proteins (kDa)
Higher plants			
Pea (*Pisum sativum*)	12.98	0.596	**30, 44, 46,64**
Bean (*Phaselous valgaris*)	2.29	0.273	**58**
Barley (*Hordeum vulgare*)	4.33	0.761	30, 58, 66
Oats (*Avena sativa*)	10.66	2.178	**28**, *80*
Pine (*Pinus taeda*)	22.02	0.551	*45*, **58**, *76*
Ginkgo (*Ginkgo biloba*)	196.78	1.850	**45**, 54, 70
Fungi			
Yeast (*S. cerevisiae*)	n.a.[b]	0.521	**110**
Neurospora crassa	6.16	0.462	*80*
Protozoa and Bacteria			
Euglena	0.18	0.005	**21, 32**
Lactobacillus casei	23.09	0.623	**33**

[a], specific enzyme activity. [b], not applicable, purified yeast C_1-THF synthase was used.

Table 11. *Immunoblot Survey of Species for Immunologically Related Proteins Using DHY-CYC-Ab.*

Preparation of enzyme extracts and SDS-PAGE were carried out as described in Table 10. The relative intensity of cross-reacting protein bands is indicated as dark (bold), median (normal), and light (italic).

SDS-treated Extracts	DHY in Extracts (nmol min^{-1} mg^{-1})	DHY Applied (nmol min^{-1} lane^{-1})	CYC in Extracts (nmol min^{-1} mg^{-1})	CYC Applied (nmol min^{-1} lane^{-1})	Cross-reacting Proteins M_r (kDa)
Pea (*Pisum sativum*)	8.691	0.029	3.22	0.011	**38**
Bean (*Phaselous vulgaris*)	0.593	0.014	n.d.[a]	n.d.[a]	**39**
Barley (*Hordeum vulgare*)	2.304	0.541	2.278	0.535	*37*
Oats (*Avena sativa*)	2.559	0.131	3.066	0.156	36
Pine (*Pinus taeda*)	2.929	0.149	7.008	0.357	39
Ginkgo (*Ginkgo biloba*)	0.421	0.016	n.d.[a]	n.d.[a]	39
Yeast (*S. cerevisiae*)	n.a.[b]	0.062	n.a.[b]	0.043	**110**
Neurospora crassa	1.126	0.134	6.750	0.803	**29**, 35
Mushroom (*A. brunnescens*)	n.d.[a]	n.d.[a]	1.530	0.032	**38**
Euglena	4.688	0.013	n.d.[a]	n.d.[a]	**38**
Lactobacillus casei	3.927	0.106	2.618	0.071	60, **73**

[a], not detected. [b], not applicable, purified yeast C_1-THF synthase was used.

plants and *Euglena*, only a single cross-reacting band was detected by SYN-Ab (Table 10).

3.5. Mitochondrial SYN, DHY and CYC Activities and the Presence of Cross-reacting Proteins in Pea Leaf Mitochondrial Extracts

3.5.1. Effect of storage on mitochondrial SYN, DHY and CYC activities

Isolated pea leaf mitochondria were stored to accumulate samples for enzyme fractionation. Due to the low levels of mitochondrial SYN, DHY and CYC activities (Table 5) and their poor stabilities, attempts to purify these mitochondrial enzymes were not successful. However, when sonicated and Triton X-100 treated mitochondrial extracts were stored at -20 °C for 14 d, both DHY and CYC activities dropped significantly, whereas the activity of SYN appeared to increase slightly (Table 12). In contrast, when extracts were prepared from mitochondrial pellets that had been stored at -20 °C for 14 d, SYN activity was not detected, while DHY and CYC remained at levels comparable to those of freshly prepared extracts (Table 12). This implies that mitochondrial SYN may be associated with a protein that has stability characteristics distinct from those of DHY or CYC.

3.5.2. Indirect competitive ELISA of mitochondrial extracts using DHY-CYC-Ab and SYN-Ab

Mitochondrial extracts subjected to SDS-PAGE were shown previously to contain

Table 12. *The Effect of Storage at -20 °C for 14 days on the Stability of Mitochondrial SYN, DHY and CYC Activities.*

Approximately 60 mg of mitochondrial protein were used for each treatment. Extracts were prepared by resuspending mitochondrial pellets in Buffer A (containing 0.1% Triton X-100) followed by sonication (see Section 2.4).

Treatments	Synthetase		Dehydrogenase		Cyclohydrolase	
	TU[a]	SA[b]	TU[a]	SA[b]	TU[a]	SA[b]
1. Freshly prepared mitochondrial extract	0.323	5.30	0.196	3.21	0.138	2.26
2. Frozen mitochondrial extract	0.452	7.41	n.a.[c]	n.d.[d]	0.014	0.23
3. Mitochondrial extract from frozen pellets	n.a.[c]	n.d.[d]	0.173	2.84	0.126	2.07

[a], total units (μmol min^{-1}); [b], specific activity (nmol min^{-1} mg^{-1}); [c], not applicable; [d], not detected.

1, extracts were assayed prior to a 14 d storage period at -20 °C.

2, extracts assayed after a 14 d storage period at -20 °C.

3, extracts were prepared from mitochondrial pellets that had been stored for 14 d at -20 °C.

proteins that cross-reacted with DHY-CYC-Ab and SYN-Ab respectively (Figures 10 and 15). It is clear from the results of indirect competitive ELISAs (Figures 17 and 18) that mitochondrial proteins in their native forms also strongly cross-reacted with DHY-CYC-Ab and SYN-Ab, respectively.

3.5.3. Immunoaffinity chromatography of mitochondrial extracts

An immunoaffinity column, containing bound DHY-CYC-Ab, was used in an attempt to fractionate the low levels of SYN, DHY and CYC activity in whole mitochondrial extracts.

When DHY-CYC-Ab was used as an immobile ligand, 90% of the DHY and CYC activities in whole mitochondrial extracts were retained by the column, whereas approximately 90% of the SYN activity passed through the column and was recovered in the column wash (Table 13). This suggests that the bulk of mitochondrial SYN is not associated, as in yeast and mammalian cells, with DHY and CYC activity. Although enzyme activities could not be detected in the neutralized Gly-HCl buffer eluant, several silver-stained protein bands were recovered (Figure 19A). Immunoblots of the column wash (Figure 19B, lane 2) and column eluant (Figure 19B, lane 3) showed that some of these proteins cross-reacted with the goat anti-rabbit secondary antibodies (Figure 19B). This indicates that some loosely coupled rabbit IgG was released from the column matrix. Despite this release, an additional cross-reacting band of approximately M_r of 38,000 was only revealed when DHY-CYC-Ab was used as primary antibody (Figure 19B and 19C).

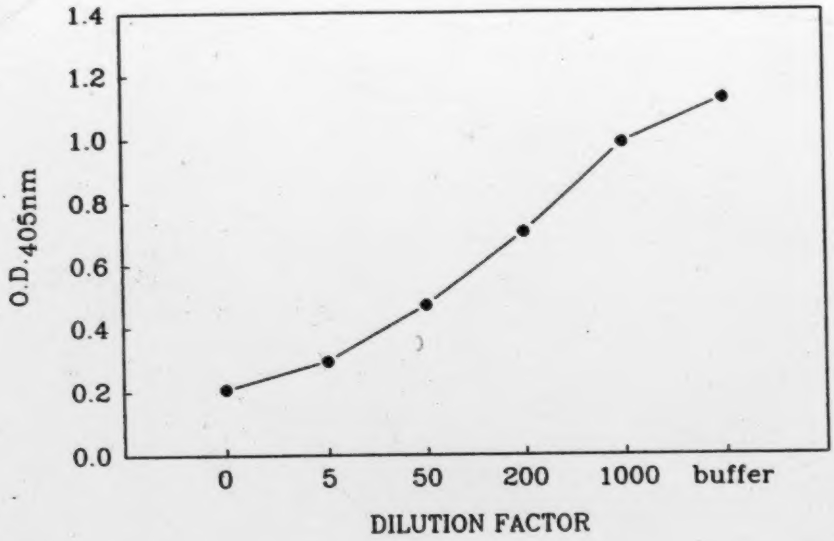


Figure 17. *Indirect Competitive ELISA of Mitochondrial Extracts Using DHY-CYC-Ab As Primary Antibody.*

Microtiter wells were coated with antigen (purified cytosolic DHY-CYC), blocked with BSA and then incubated with equal volumes of diluted test extract and diluted purified rabbit IgG. Secondary antibodies were Sigma goat anti-rabbit IgG conjugated with alkaline phosphatase. Controls (indicated by buffer values) had all treatments but lacked the mitochondrial extract which was replaced with buffer. Prior to ELISA, mitochondrial extracts were prepared as described in Materials and Methods. Each point represents the mean of triplicate values with a standard error typically less than 5%.

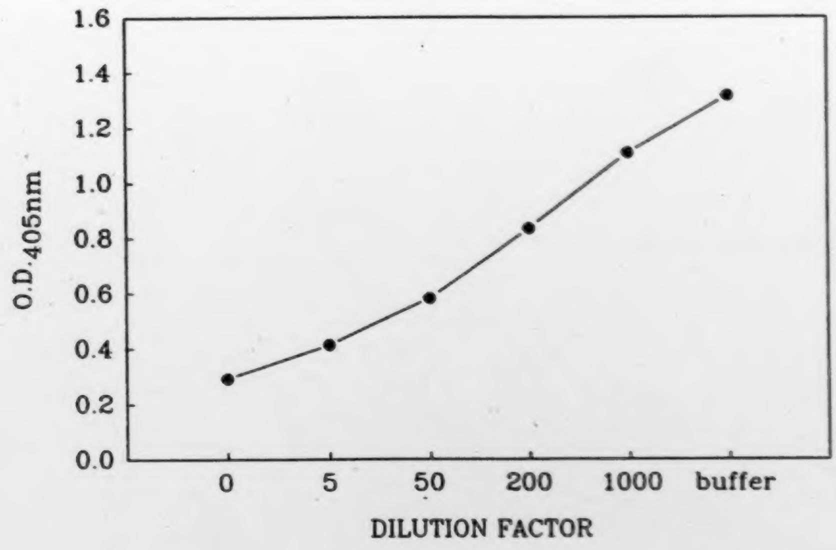


Figure 18. *Indirect Competitive ELISA of Mitochondrial Extracts Using SYN-Ab As Primary Antibody.*

Microtiter wells were coated with antigen (purified cytosolic SYN), blocked with BSA and then incubated with equal volumes of diluted test extract and diluted purified rabbit IgG. Secondary antibodies were Sigma goat anti-rabbit IgG conjugated with alkaline phosphatase. Controls (indicated by buffer values) had all treatments but lacked the mitochondrial extract which was replaced with buffer. Prior to ELISA, mitochondrial extracts were prepared as described in Materials and Methods. Each point represents the mean of triplicate values with a standard error typically less than 5%.

Table 13. *Immunoaffinity Chromatography of Mitochondrial SYN, DHY and CYC Activities*

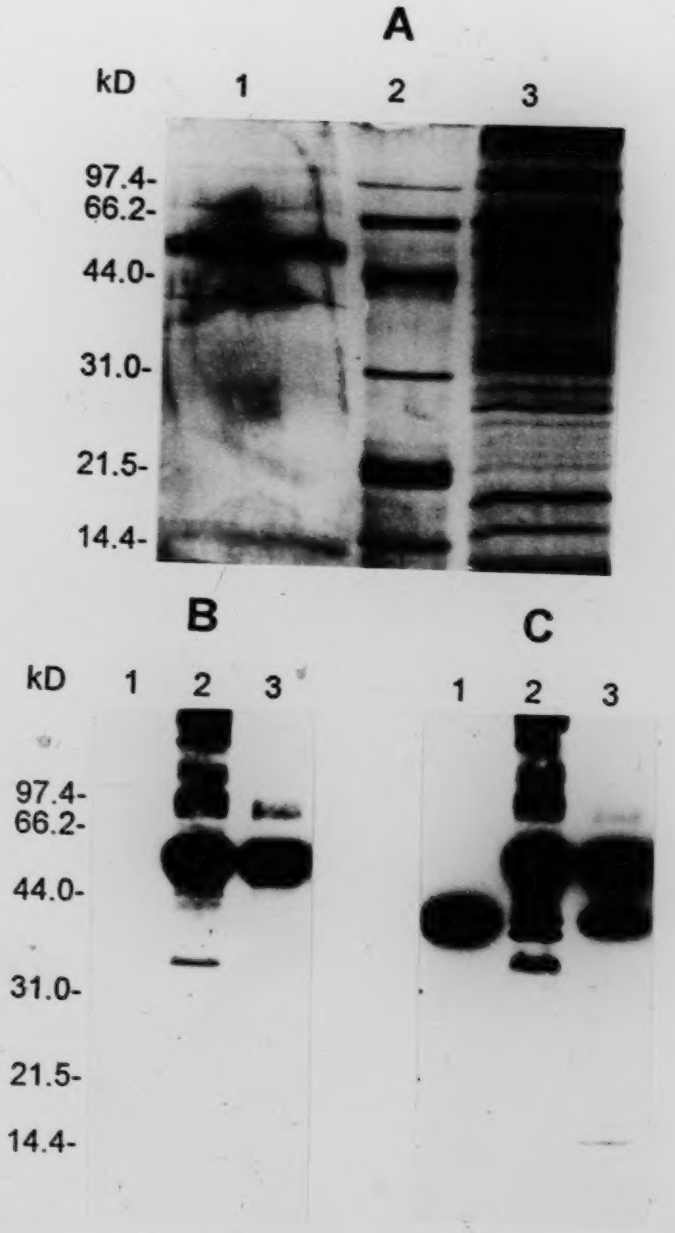
Total activities (SYN, 0.424 μmol min^{-1}; DHY, 0.257 μmol min^{-1}; CYC, 0.181 μmol min^{-1}), present in the initial mitochondrial extract (5 ml), were used to calculate the percentages of each activity recovered in the column fractions. After application of the mitochondrial extract, the affinity column, containing DHY/CYC-Ab as an immobile ligand, was washed with 5 ml of the loading buffer to obtain "column wash" fractions followed by washing with GuHCl and 0.2 M Gly-HCl (pH 2.8) to obtain "eluant" fractions (see Section 2.8).

Fractions recovered	SYN (%)	DHY (%)	CYC (%)
Initial extract	100	100	100
Column wash	90	8.3	9.4
Eluant fractions	n.d.[a]	n.d.[a]	n.d.[a]

[a], activity not detected by the standard enzyme assay procedure.

Figure 19. *SDS-PAGE (A) and Immunoblots (B and C) of Mitochondrial Proteins before and after Immunoaffinity Chromatography.*

The polyclonal antibodies (DHY-CYC-Ab) were raised against purified, cytosolic DHY-CYC protein (see Section 2.7). A, Mitochondrial proteins that were bound and then eluted from the immunoaffinity column (lane 1); molecular weight standards (lane 2); whole mitochondrial extract before chromatography (lane 3). B, Western blot analyses in which the primary antibody incubation step with DHY-CYC-Ab was omitted. C, Western blots using DHY-CYC-Ab as primary antibody. Whole mitochondrial extract before immunoaffinity chromatography (B and C, lane 1); mitochondrial proteins that passed through the column and were collected in the initial column wash (B and C, lane 2); and proteins that were retained by the column and then eluted with Gly-HCl buffer pH 2.8 (B and C, lane 3).


A
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97.4-
66.2-
44.0-
31.0-
21.5-
14.4-
B
kD
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2
3
97.4-
66.2-
44.0-
31.0-
21.5-
14.4-
C
1
2
3

3.7. The Effect of Greening on the Expression of DHY, CYC and SYN

It is clear that the total folate pool is enlarged when etiolated leaves are exposed to light (Spronk and Cossins, 1972). In addition, during the development of photorespiration in greening tissues, the expression of two folate-dependent enzymes, SHMT and GDC, is also strongly up-regulated (Turner et al., 1993; Walker and Oliver, 1986; Oliver and Raman, 1995). It therefore would be of interest to examine the greening effect on the expression of SYN, DHY and CYC, enzymes that might utilize part of the 5,10-CH_2-$H_4PteGlu_n$ arising from the photorespiratory cleavage of glycine. When 9 d etiolated pea seedlings were exposed to light for a period of 72 h, the specific activity of SYN in whole leaf extracts dropped 4-fold, whereas the specific enzyme activities of DHY and CYC rose about 2-fold (Figure 20). Immunoblots suggested that greening also affected the amounts of cross-reacting protein (Figure 21 and 22). Thus the amount of SYN-Ab cross-reacting protein, as judged by immunoblot analyses, decreased dramatically in extracts of plants exposed to light (Figure 21). A smaller but significant decrease in SYN-Ab cross-reacting 66 kDa protein also occurred in plants maintained in the dark (Figure 21). Thus the levels of this protein may decline during seedling development. In contrast, the amount of DHY-CYC-Ab cross-reacting protein in whole-leaf extracts increased during greening (Fig. 22A). The decreases noted for SYN-Ab reacting protein appeared to include the 44 kDa band associated with mitochondrial extracts (Figure 15). Assay of mitochondrial extracts, prepared from etiolated and greening seedlings (Table 14), showed that the specific activity of SYN decreased by more than 2-fold following 48 h of illumination but

Figure 20. *The Effect of Greening on Specific Enzyme Activities.*

9 d, dark-grown plants were exposed to light (open symbols) for the periods indicated or maintained in the dark throughout (closed symbols, controls). Whole-leaf extracts were assayed. Each point is the mean of three separate determinations with standard error between 8-13%. Specific activities are expressed in nmol min^{-1} mg^{-1}.

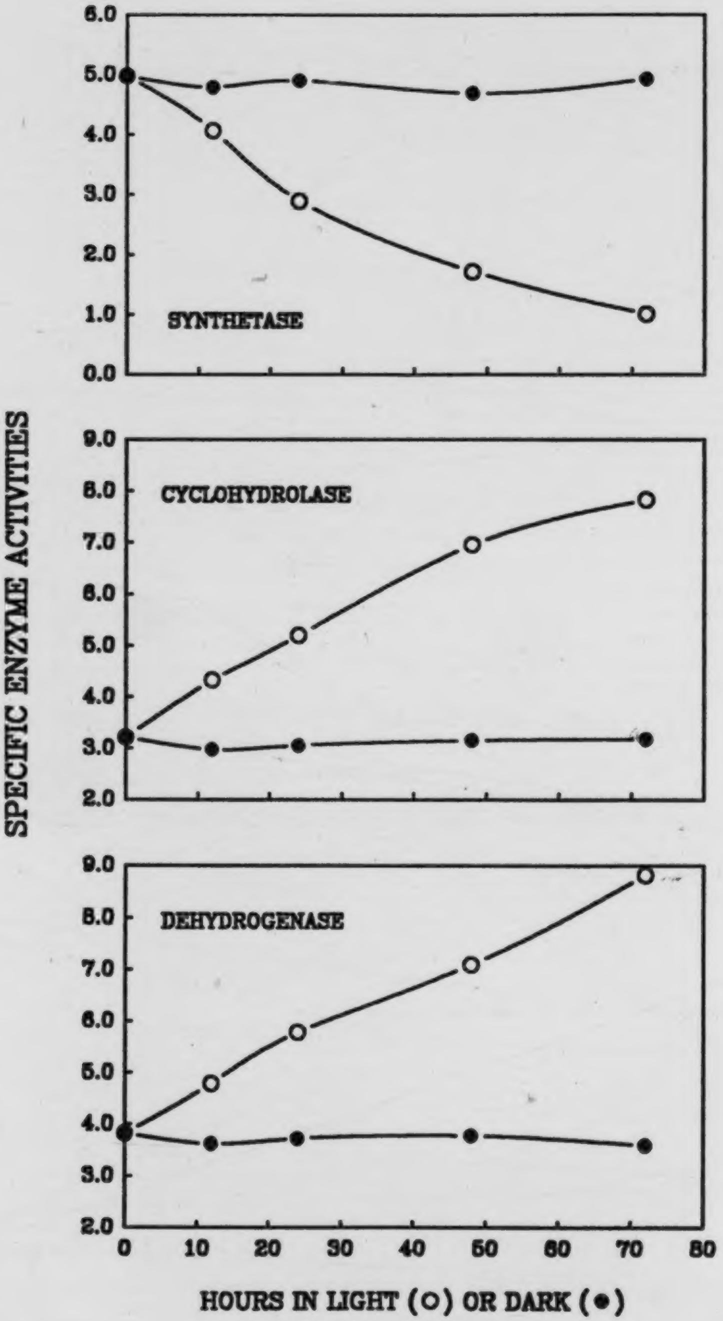
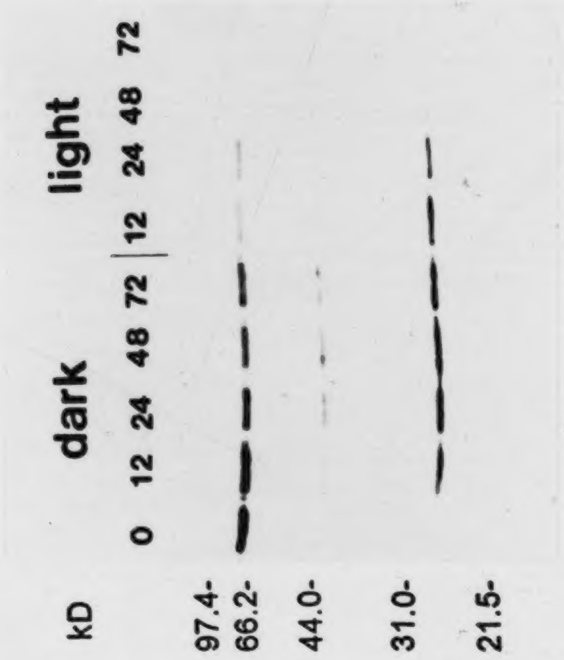

SPECIFIC ENZYME ACTIVITIES
SYNTHETASE
6.0
5.0
4.0
3.0
2.0
1.0
0.0
CYCLOHYDROLASE
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
DEHYDROGENASE
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
0
10
20
30
40
50
60
70
80
HOURS IN LIGHT (○) OR DARK (●)

Figure 21. *Immunoblots of SYN Protein in Extracts of Light-treated Etiolated Pea Leaves.*

The numbers above each lane indicate hours of light or dark exposure of seedlings that were initially grown in darkness for 9 d. 20 µg of protein were loaded in each lane.


kD
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light
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24
48
72
12
24
48
72
97.4-
66.2-
44.0-
31.0-
21.5-

Figure 22. *Immunoblots of DHY-CYC Protein in Extracts of Light-treated Etiolated Leaves.*

(A) whole-leaf extracts; 20 µg protein loaded per lane; (B) mitochondrial extracts of greening leaves; 12 µg protein loaded per lane. The numbers above each lane indicate hours of light or dark exposure of seedlings initially grown in darkness for 9 d. M0, mitochondrial extract prepared from leaves of plants grown in darkness for 9 d. Leaf mitochondrial extracts of 9 d, dark-grown plants that had been maintained in darkness for an additional 48 h (MD48), or placed in the light for 48 h (ML48).

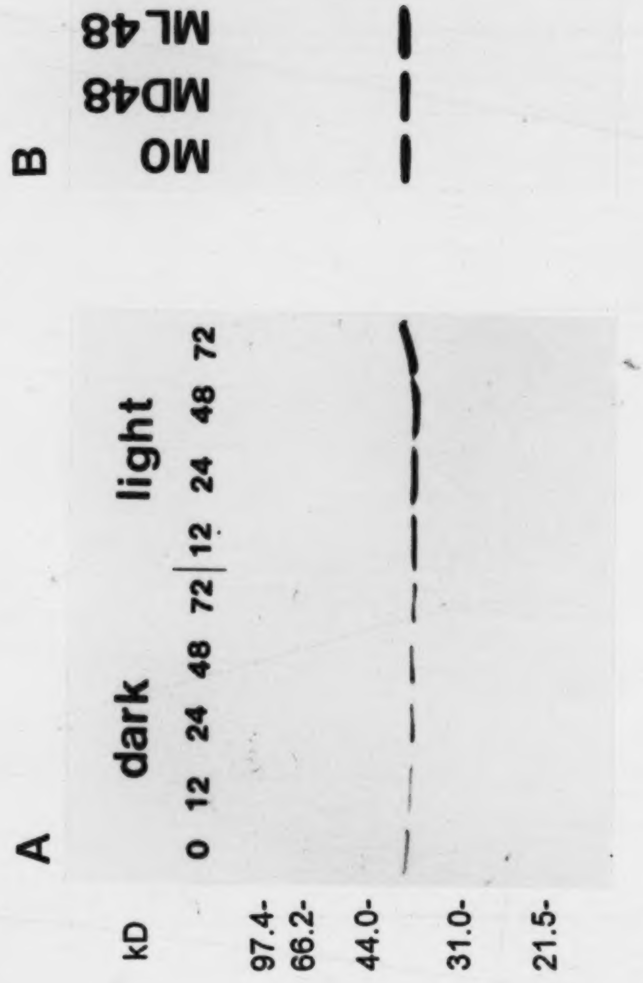
B
MO
MD48
ML48
A
dark
light
0
12
24
48
72
12
24
48
72
kD
97.4-
66.2-
44.0-
31.0-
21.5-

Table 14. *The Effect of Greening on the Specific Activities (nmol min^{-1} mg^{-1}) of Mitochondrial SYN, DHY and CYC.*

Seedlings were grown for 9 d in darkness and then transferred to continuous light for 48 h. Controls were maintained in the dark for a total period of 11 d. Mitochondria were isolated, purified and suspended in 25 mM HEPES buffer (pH 7.5), containing 0.1% Triton X-100, 1 mM PMSF, 10 mM 2-mercaptoethanol, 10 mM KCl, and 20% glycerol and assayed for each enzyme activity (see Materials and Methods). Data are the averages of three separate determinations (± standard errors).

Mitochondrial extracts	SYN	DHY	CYC
Dark-grown, 9 d	3.73 ± 0.41	3.54 ± 0.29	4.48 ± 0.56
Dark-grown, 11 d	3.93 ± 0.35	3.36 ± 0.35	4.62 ± 0.60
Light-grown, 48 h[a]	1.11 ± 0.12	2.89 ± 0.24	4.07 ± 0.43

[a], 9 d etiolated plants were transferred to light for 48 h.

the levels of DHY and CYC were not appreciably changed. Immunoblots of these mitochondrial extracts suggested that the amounts of DHY-CYC cross-reacting protein remained approximately the same before and after the light treatment (Figure 22B).

3.6. Isolation and Characterization of a cDNA Encoding Cytosolic DHY-CYC

Isolation of a cDNA encoding cytosolic DHY-CYC

DHY-CYC-Ab was used to screen a 10 d light-grown pea leaf λgt11 cDNA expression library. This yielded a 830 bp cDNA insert fragment from a clone designated Dλ2. This cDNA encoded a partial amino acid sequence that was prematurely truncated downstream of the N-terminus of the coding sequence (Figure 23). This assessment was based on: a) lack of a start methionine; and b) comparisons with the published sequences of the protein from other species (Staben and Rabinowtz, 1986; Shannon and Rabinowitz, 1988; D'Ari and Rabinowitz, 1991; etc.). The insert of Dλ2 was therefore used as a probe for further screening (see Section 2.16) and a nucleotide sequence of 1105 bp was obtained from a phage clone designated DD7 (Figure 23). The insert in DD7 also appeared to be truncated prematurely. It was therefore decided to use the most 5′ region of this clone as a probe for further screening so as to maximize the chances of finding full length clones. The DD7 insert was amplified by PCR and digested with Hind III. This gave rise to a 377 bp sequence from the 5′ end. A 9 d dark-grown pea leaf λgt11 library was screened using the 377bp sequence as a probe. This resulted in the isolation of a

Figure 23. Nucleotide and Corresponding Amino Acid Sequences of a 1.2 kb cDNA Clone (D3-1) Encoding Cytosolic DHY-CYC .

The nucleotide sequence is numbered sequentially starting from the first nucleotide. The deduced amino acid sequence starts from the first methionine and ends before the stop codon. The 5′ and 3′ ends of the Dλ2 and DD7 clones are indicated by (⌈) and (⌉) respectively. Stop codons are marked by asterisks. The position of a potential poly(A) site is underlined.

```
                                    AAGGACA AGTCGTGAAG         17

TTGTATTTTA ACAGAAAACC ACACCGCACG ATCTCAGTTT CACCGATCAA         67
        ** *
                                                    ⌈DD7
ATG GCC ACC GTA ATC GAC GGC AAA GCC GTT GCA CAA ACT ATC CGA   112
MET Ala Thr Val Ile Asp Gly Lys Ala Val Ala Gln Thr Ile Arg

TCT GAA ATC GCC GAC GAG GTT CGT CTT CTC TCA CAA AAG TAC GGC   157
Ser Glu Ile Ala Asp Glu Val Arg Leu Leu Ser Gln Lys Tyr Gly

AAG GTT CCT GGA CTT GCA GTG GTG ATA GTA GGA AAC CGA AAG GAC   202
Lys Val Pro Gly Leu Ala Val Val Ile Val Gly Asn Arg Lys Asp
                                                         ⌈Dλ2
TCT CAA AGC TAC GTT GGA ATG AAG AGA AAA GCG TGT GCT GAA TTG   247
Ser Gln Ser Tyr Val Gly MET Lys Arg Lys Ala Cys Ala Glu Leu

GGA ATC AAA TCC TTC GAT ATC GAC CTT CCG GAG GAT GCT TCT GAA   292
Gly Ile Lys Ser Phe Asp Ile Asp Leu Pro Glu Asp Ala Ser Glu

GCT GAA ATC ATA AAA AAT GTT CAC GAA TTG AAC GCT AAC CCC GAT   337
Ala Glu Ile Ile Lys Asn Val His Glu Leu Asn Ala Asn Pro Asp

GTA CAT GGT ATA TTG GTT CAA CTT CCA TTG CCT AAG CAC GTA AAT   382
Val His Gly Ile Leu Val Gln Leu Pro Leu Pro Lys His Val Asn

GAA GAG AAA GTT TTG ACT GAA ATC AGC ATT TCA AAG GAT GTA GAT   427
Glu Glu Lys Val Leu Thr Glu Ile Ser Ile Ser Lys Asp Val Asp

GGC TTC CAT CCC TTG AAC ATT GGC AAG CTT GCA ATG AAA GGA AGA   472
Gly Phe His Pro Leu Asn Ile Gly Lys Leu Ala MET Lys Gly Arg

GAC CCT CTG TTT CTT CCA TGT ACT CCC AAG GCA TGT CTT GAA CTA   517
Asp Pro Leu Phe Leu Pro Cys Thr Pro Lys Ala Cys Leu Glu Leu

TTA TCA CGA AGT GGT GTA AGT ATA AAG GGG AAA AAG GCT GTT GTG   562
Leu Ser Arg Ser Gly Val Ser Ile Lys Gly Lys Lys Ala Val Val

GTT GGT AGA AGC AAC ATA GTT GGA TTA CCA GCT TCA TTG CTG CTT   607
Val Gly Arg Ser Asn Ile Val Gly Leu Pro Ala Ser Leu Leu Leu

CTG AAA GCA GAT GCT ACA GTT ACC ATT GTT CAT TCA CAC ACA AGT   652
Leu Lys Ala Asp Ala Thr Val Thr Ile Val His Ser His Thr Ser
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CAA CCA GAA ACT ATC ATT CGT GAA GCA GAT ATT GTT ATT GCA GCA      697
Gln Pro Glu Thr Ile Ile Arg Glu Ala Asp Ile Val Ile Ala Ala

GCA GGA CAG GCA AAG ATG ATC AAG GGA AGC TGG ATA AAA CCA GGA      742
Ala Gly Gln Ala Lys MET Ile Lys Gly Ser Trp Ile Lys Pro Gly

GCT GCA GTG ATA GAT GTT GGC ACA AAT TCT GTG GAT GAC CCA ACT      787
Ala Ala Val Ile Asp Val Gly Thr Asn Ser Val Asp Asp Pro Thr

AGG AAA TCA GGT TAT AGA CTT GTT GGA GAT GTA GAT TTT GAG GAA      832
Arg Lys Ser Gly Tyr Arg Leu Val Gly Asp Val Asp Phe Glu Glu

GCA TCT AAA GTT GCT GGT TGG ATT ACT CCT GTT CCT GGT GGT GTG      877
Ala Ser Lys Val Ala Gly Trp Ile Thr Pro Val Pro Gly Gly Val

GGT CCA ATG ACA GTC ACA ATG TTG CTG AAG AAT ACT TTG GAG GGC      922
Gly Pro MET Thr Val Thr MET Leu Leu Lys Asn Thr Leu Glu Gly

GCT AAA CGC ACC ATT GAG CAG AAT AAC TAAATATTCC TTTAGTTTTA        969
Ala Lys Arg Thr Ile Glu Gln Asn Asn ***

ATAAAAAGAA AGTTACTCTT CGTTTGAGAA TCAGTTTTTG CTGATAGATC          1019

AATGTATCTT TATAAAACCG TAAGAATTGG TATTGAAAGT TGTTTTTGGT          1069

  Dλ2]
TCTTCTCGTT CATTATTTTA GTTTCTCCCC CTTATAGTTA CCTTGTTCTC          1019

TTTCTTGTAG ACTTATATTG GAATGCCGTC AAGTCTGGTA AGGCTTCAAA          1169

                                            DD7]
TAGTACACCA AAATAAGGGA ACTATTATGA TGTTAATGTC AAACCGGCCC          1219
```

phage clone designated D3-1 containing an insert of 1219 bp that appeared to encode the full-length amino acid sequence of DHY-CYC (Figure 23).

The derived amino acid sequence of D3-1 contained 294 amino acid residues from the first methionine to the stop codon (Figure 23). The molecular weight of this translated sequence was calculated to be 31,344 Da and the estimated isoelectric point was equal to 8.46. As a homodimeric organization was suggested for this enzyme in Section 3.2.1, this calculated subunit molecular weight is consistent with the native M_r of 58,000-60,000 derived from gel filtration studies of this protein (Figures 7 and 8). Conceivably, the relatively high positive charge of this protein would retard its mobility on SDS-PAGE gels and thus result in an erroneously high value (38,000) for the subunit M_r (Figure 6). Figure 23 shows that a stop codon (TAA) occurs 42 bp upstream of the start codon. This suggests that the upstream sequence does not encode an organellar targeting sequence.

Characterization of D3-1 and its derived amino acid sequence

When total RNA isolated from pea leaves was probed with ^{32}P-labeled Dλ2 insert, a single mRNA band of 1.2 kb was revealed by Northern blot analyses (Figure 24). This value was in agreement with the size of the D3-1 nucleotide sequence, that encoded the full-length amino acid sequence of the cytosolic pea DHY-CYC (Figure 23).

The amino acid sequence derived from D3-1 was aligned with sequences of the DHY-CYC bifunctional enzymes or the DHY/CYC domains of trifunctional C_1-THF synthases of *E. coli*, yeast or mammalian cells (Figure 25). Based on these alignments, the primary structure of pea DHY-CYC shares most of the conserved domains that are found

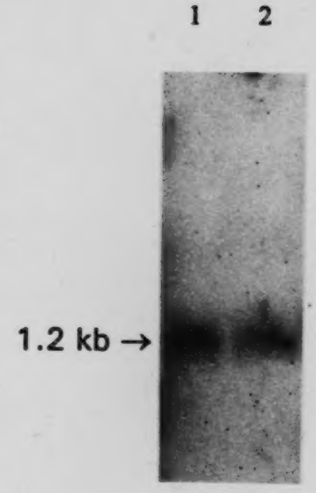

Figure 24. *Northern Blot of Total RNA from Pea Leaves*

A ^{32}P-labeled cDNA insert fragment from Dλ2 (subcloned in phagemids pBluescript SK (+/-), see Section 2.16) was used as a probe. Promega RNA ladder markers were run concurrently to allow size estimates. Kodak XAR films were exposed with a intensifying screen at -70 °C for two weeks. Total RNA, 12 μg and 15 μg (lane 1 and lane 2) were loaded.

Figure 25. Alignment of the Deduced Amino Acid Sequences of Eight Dehydrogenase-Cyclohydrolase Domains.

The abbreviations for the sources of the enzymes shown are: HUM, human; YMI, yeast mitochondria; YCY, yeast cytoplasm; ECO, *E,coli*; MOU, mouse. The enzymatic activities associated with the bi- and tri-functional enzymes listed are: S, 10-formyl-THF synthetase; D, 5,10-methylene-THF dehydrogenase; C, 5,10-methenyl-THF cyclohydrolase. The (!) indicates a stop codon. The numerals indicate the position of the amino acid in the enzyme sequence as reported in the data bank. The amino acids with an (*) underneath are common to all eight sequences, and those with an (^) underneath are present in all seven sequences but not in PEA DC.

```
PEA  DC       MATVIDGKAV.AQTIRSEIA.DEVRLLSQKYGKVPGLAVVIVGNRKDSQ      47
HUM SDC     MAPAEILNGKEISAQIRARLKN.QVTQLKEQVPGFTPRLAILQVGNRDDSN      50
RAT SDC     MAPAGILNGKVVSAQIRNLLKT.QFTQMQEQVPGFTPGLAILQVGDRDDSN      50
YMI SDC      SQYHILSGRKL.AQSIREKANDEIQAIKLKHPNFKPTLKIIQVGARPDSS      81
YCY SDC      MAGQVLDGKAC.AQQFRSNIANEIKSIQGHVPGFNPALAIIQVGNRPDSA      49
ECO  DC      MAAKIIDGKTI.AQQVRSEVA.QKVQARIAAGLRAPGLAVVLVGSNPASQ      48
HUM  DC      NEAVVISGRKL.AQQIKQEVR.QEVEEWVASGNKRPHLSVILVGENPASH      80
MOU  DC      NEAVVISGRKL.AQQIKQEVQ.QEVEEWVASGNKRPHLSVILVGDNPASH      82
                    *    **                     * *    **    *

PEA  DC      SYVGMKRKACAELGIKSFDIDLPEDASEAEIIKNVHELNANPDVHGILVQ      97
HUM SDC      LYINVKLKAAEEIGIKATHIKLPRTTTESEVMKYITSLNEDSTVHGFLVQ     100
RAT SDC      LYINVKLKAAQEIGIKATHIKLPRTSTESEVLKYVISLNEDATVHGFIVQ     100
YMI SDC      TYVRMKLKASKDSNVDCIIEKLPAEITEVELLKKISDINDDDSIHGLLIQ     131
YCY SDC      TYVRMKRKAAEEAGIVANFIHLDESATEFEVLRYVDQLNEDPHTHGIIVQ      99
ECO  DC      IYVASKRKACEEVGFVSRSYDLPETTSEAELLELIDTLNADNTIDGILVQ      98
HUM  DC      SYVLNKTRAAAVVGINSETIMKPASISEEELLNLINKLNNDDNVDGLLVQ     130
MOU  DC      SYVLNKTRAAAEVGINSETIVKPASVSEEELLNSIRKLNNDENVDGLLVQ     132
              *   *  *             *    * *        *      *   *

PEA  DC      LPLPKH..VNEEKVLTEISISKDVDGFHPLNIGKLAMKGRDPLFLPCTPK     145
HUM SDC      LPLDSENSINTEEVINAIAPEKDVDGLTSINAGRLARGDLNDCFIPCTPK     150
RAT SDC      LPLDSENSINTEAVINAIAPEKDVDGLTSINAGKLARGDLKDCFIPCTPK     150
YMI SDC      LPLPRH..LDETTITNAVDFKKDVDGFHRYNAGELAKKGGKPYFIPCTPY     179
YCY SDC      LPLPAH..LDEDRITSRVLAEKDVDGFGPTNIGELNKKNGHPFFLPCTPK     147
ECO  DC      LPLPAG..IDNVKVLERIHPDKDVDGFHPYNVGRLCQRAPR..LRPCTPR     144
HUM  DC      LPLPEH..IDERRICNAVSPDKDVDGFHVINVGRMCLDQYS..MLPATPW     176
MOU  DC      LPLPEH..IDERKVCNAVSPDKDVDGFHVINVGRMCLDQYS..MLPATPW     178
             ***                  *****    * *            * **

PEA  DC      ACLELLSRSGVSIKGKKAVVVGRSNIVGLPASLLLLKA........DATV     187
HUM SDC      GCLELIKETGVPIAGRHAVVVGRSKIVGAPMHDLLLWN........NATV     192
RAT SDC      GCLELIKETGVQIAGRHAVVVGRSKIVGAPMHDLLLWN........NATV     192
YMI SDC      GCMKLLEEAHVKLDGKNAVVLGRSSIVGNPIASLLKNA........NATV     221
YCY SDC      GIIELLHKANVTIEGSRSVVIGRSCIVGSPVAELLKSL........NSTV     189
ECO  DC      GIVTLLERYNIDTFGLNAVVIGASNIVGRPMSMELLLA........GCTT     186
HUM  DC      GVWEIIKRTGIPTLGKNVVVAGRSKNVGMPIAMLLHTDGAHERPGGDATV     226
MOU  DC      GVWEIIKRTGIPTLGKNVVVAGRSKNVGMPIAMLLHTDGAHERPGGDATV     228
             ^             *   ** * *  ** *    *             *

PEA  DC      TIVHSHTS..QPETIIREADIVIAAAGQAKMIKGSWIKP........GAA     227
HUM SDC      TTCHSKTA..HLDEEVNKGDILVVATGQPEMVKGEWIKP........GAI     232
RAT SDC      TTCHSKTA..DLDKEVNKGDILVVATGQPEMVKGEWIKP........GAV     232
YMI SDC      TVCHSHTR..NIAEVVSQADIVIAACGIPQYVKSDWIKE........GAV     261
YCY SDC      TITHSKTR..DIASYLHDADIVVVAIGQPEFVKGEWFKPRDGTSSDKKTV     237
ECO  DC      TVTHRFTK..NLRHHLENADLLIVAVGKPGFIPGDWIKE........GAI     226
HUM  DC      TISHRYTPKEQLKKHTILADIVISAAGIPNLITADMIKE........GAA     268
MOU  DC      TISHRYTPKEQLKKHTILADIVISAAGIPNLITADMIKE........GAA     270
             *  *  *            *    * * ^
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PEA  DC    VIDVGTNSVDDPTRKSGYRLVGDVDFEEASKVAGWITPVPGGVGPMTVTM    277
HUM SDC    VIDCGINYVPDDKKPNGRKVVGDVAYDEAKERASFITPVPGGVGPMTVAM    282
RAT SDC    VIDCGINYVPDDTKPNGRKVVGDVAYDEAKEKASFITPVPGGVGPMTVAM    282
YMI SDC    VIDVGINYVPDDTKPNGRKVVGDVAYDEAKEKASFITPVPGGVGPMTVAM    311
YCY SDC    VIDVGTNYVADPSKKSGFKCVGDVEFNEAIKYVHLITPVPGGVGPMTVAM    287
ECO  DC    VIDVGINRLENG......KVVGDVVFEDAAKRASYITPVPGGVGPMTVAT    270
HUM  DC    VIDVGINRVHDPVTAKP.KLVGDVDFEGVRQKAGYITPVPGGVGPMTVAM    317
MOU  DC    VIDVGINRVQDPVTAKP.KLVGDVDFEGVKKKAGYITPVPGGVGPMTVAM    319
           *** * *           ^ ****           *************^

PEA  DC    LLKNTLEGAKRTIEQNN!                                    294
HUM SDC    LMQSTVESAKRFLEKFKPGKWMIQYNNLNLKTP                     315
RAT SDC    LMQSTVESAQRFLKKFKPGKWTIQYNKLNLKTP                     315
YMI SDC    LVSNVLLAAKRQFVESEKLP.VIKPLPLHLESP                     343
YCY SDC    LMQNTLIAAKRQMEESSKPL.QIPPLPLKLLTP                     319
ECO  DC    LIENTLQACVEYHDPQDE!                                   288
HUM  DC    LMKNTIIAAKKVLRLEEREVLKSKELGVATN!                      348
MOU  DC    LMKNTIIAAKKVLRPEELEVFKSKQRGVATN!                      350
           *
```

in the corresponding enzymes of other sources. There were however four amino acids in pea protein (Ala-146, Ala-214, Arg-246 and Thr-276), which were not found in the conserved amino acid residues shared by the other seven species.

The cytosolic and mitochondrial forms of C_1-THF synthase in yeast cells are encoded by separate nuclear genes (Staben and Rabinowitz, 1986; Shannon and Rabinowitz, 1988). When the DHY/CYC domains of the protein encoded by these genes were compared, 52% identity of the amino acid sequences was found (Shannon and Rabinowtz, 1988). The alignment of the predicted amino acid sequence of pea cytosolic DHY-CYC with the corresponding domains of these yeast proteins shows identities of 46% and 52% respectively for the cytosolic and mitochondrial forms (Table 15). Interestingly, the lowest identity of 25% was found when pea DHY-CYC was compared with the primary structure of the yeast NAD-dependent, mono-functional dehydrogenase.

Table 15. *Percentage Identity Shown Between the Amino Acid Sequence of Pea DHY-CYC and Those of Corresponding Enzymes or Domains of Other Species.*

Source	% Identity
Pea bi-functional DHY-CYC	
Human C_1-THF synthase	49.0
Human bi-functional DHY-CYC	46.3
Rat C_1-THF synthase	49.0
Mouse bi-functional DHY-CYC	48.3
Yeast mitochondrial C_1-THF synthase	46.3
Yeast cytosolic C_1-THF synthase	52.4
Yeast NAD-dependent dehydrogenase	25.2
E. coli bi-functional DHY-CYC	49.0

4. DISCUSSION

The seven areas presented in the Results section will now be discussed. The association of the cytosolic DHY and CYC activities and the existence of a separate mono-fuctional SYN activity in pea leaves will be discussed together, whereas the remaining five topics are discussed separately. In the discussion of each section, suggestions are made for future studies that would provide more new information.

4.1. The Intracellular Distribution of SYN, DHY, and CYC Activities in Pea Leaves

The occurrence of SYN, DHY, and CYC activities in higher plants has been reported by a number of laboratories (for review see Cossins, 1980). It is therefore not surprising that these three folate-dependent enzymes were readily detected in whole-leaf extracts of pea seedlings when the standard assay conditions were followed (Table 2). As noted earlier (Sections 1.2.7; 1.3.3; 1.3.4), in yeast and mammalian cells, SYN, DHY and CYC occur in the cytosolic and mitochondrial compartments, and these activities are accompanied by a corresponding subcellular distribution of folate derivatives (Appling, 1991; Wagner, 1996). In higher plants, the relatively large cytosolic folate pool is accompanied by a variety of folates associated with the mitochondria and chloroplasts (Cossins and Shah, 1972; Coffin and Cossins, 1986; Chen et al., 1997). It is therefore conceivable that these three folate-dependent enzymes may also occur in these organelles.

This possibility was examined in the present work by using Percoll-gradient purified organelles.

A protocol developed by Douce et al. (1987) was used to isolate mitochondria from pea leaves. This widely used method employs a self-generating gradient of Percoll in combination with linear gradients of PVP-25 and raffinose. Previous work has shown that it is suitable for the large-scale preparation of intact, active, and chlorophyll-free mitochondria from pea leaves (Douce et al., 1987). Another reliable method, which also uses a Percoll gradient (Schuler and Zielinski, 1989), was employed to isolate intact chloroplasts from pea leaves. The intactness of the isolated organelles was assessed by the assay of appropriate marker enzymes (Coffins and Cossins, 1986; Kagawa, 1982).

Only when these leaf mitochondria were sonicated in the presence of Triton X-100, were SYN, DHY, and CYC activities detected (Table 3). Interestingly, the specific activities of mitochondrial extracts, assayed according to the standard methods, were closely comparable to those of whole-leaf extracts (Tables 2 and 3). Despite this, the bulk of the recovered activities were cytosolic (Table 5). The requirement for sonication and detergent treatments to reveal activities in the mitochondria suggests that these activities are latent and possibly associated with the inner mitochondrial membrane (Table 3). This possibility is also supported by the observation that in whole-leaf extracts, the Triton X-100 treatment gave no significant increase in these activities (Table 2). On the other hand, sonication and Triton X-100 treated pea leaf chloroplast extracts failed to reveal SYN, DHY or CYC activities, or cross-reacting proteins (Figures 11 and 16). Like mitochondria, these organelles have a pool of 10-HCO-H_4PteGlu (Cossins and Shah, 1972), utilize this folate in the transformylation of methionyl-tRNA (Staben and Rabinowitz, 1984; Cossins, 1987), convert formate to serine (Shingles et al., 1984) and have SHMT activity (for review see Cossins, 1980). Thus pea chloroplasts should also have the enzymes to generate 10-HCO-, 5,10-CH^+- and 5,10-CH_2-$H_4PteGlu_n$. In this regard, Neuburger et al. (1996) recently detected DHY activity in isolated pea chloroplasts. The failure to detect these proteins in chloroplast extracts in the present study suggests that they may be structurally and catalytically distinct from their cytosolic counterparts.

The intracellular distribution of SYN, DHY and CYC in pea leaves (Table 5) shows that these activities occur mainly in the cytosol, with less than 1% of each activity present in the mitochondria. This distribution is similar to that shown by folates of pea leaves. Thus Imeson et al. (1990) showed that the folates of pea leaves are highly glutamyl conjugated and microbiological assays (Chen et al., 1997) indicated that most of this folate is cytosolic with less than 1% of leaf folates being associated with the mitochondrial fraction. In a recent study, Neuburger et al. (1996) used a radioassay procedure to examine the intracellular distribution of folates in pea leaves. Although these workers obtained similar folate levels in mitochondria as compared to those determined by Chen et al. (1997), they concluded that the mitochondrial compartment accounted for most of the cellular folate. The reason for this discrepancy may include significant losses of cytosolic folate during extract concentration and the fact that radioassays are generally less sensitive than microbiological assays (Cossins, 1984). It has also been reported that radioassays have limited value in the measurement of folylpolyglutamates (Shane et al., 1980). In higher plants, if all cellular folates are synthesized *de novo* by the mitochondria (Neuburger et al., 1996; Rebeille et al., 1997), it follows that precursors of the larger, cytosolic folate pool must be transported across mitochondrial membranes. This possibility warrants further study.

In the cytosol of pea leaves, 5-CH_3-H_4PteGlu$_n$ accounts for more than 50% of the recovered folates, while 10-HCO-H_4PteGlu$_n$ accounts for about 27% (Chen et al., 1997). As the bulk of SYN, DHY and CYC activities occur in the cytosol (Table 5), it follows that this compartment may be a major site for the interconversion of 10-HCO-H_4PteGlu$_n$ and 5,10-CH_2-H_4PteGlu$_n$. This is certainly the case in yeast where Pasternack et al. (1992, 1994b) used ^{13}C NMR to examine the metabolic flux through SHMT, GDC and C_1-THF synthase. These workers demonstrated that although mitochondrial and cytosolic isozymes of both C_1-THF synthase and SHMT exist in these cells, about 90% of the total utilization of formate for serine synthesis occurs via the cytosolic form of C_1-THF synthase.

4.2. The Structural Organization of Cytosolic SYN, DHY and CYC in Pea Leaves

When whole-leaf extracts were fractionated by column chromatography on Matrex Green A (Figure 5) in the presence of PMSF, the DHY and CYC activities were clearly separated from SYN. SDS-PAGE and silver staining suggested that DHY-CYC and SYN were purified to apparent homogeneity (Figures 6 and 12). The levels of recovered enzyme activity in these fractionations (Tables 6 and 7) suggest that both purified proteins were mainly of cytosolic origin.

The co-purification of DHY and CYC (Table 6) indicates that these activities are associated as reported for pea cotyledons (Kirk et al., 1995). The dehydrogenase of this complex was exclusively NADP-dependent as no enzyme activity was detected when assays were performed for NAD-dependent 5,10-CH_2-H_4PteGlu dehydrogenase. The native molecular weight of this DHY-CYC protein (Figures 7 and 8) was about 58 to 60 kDa. Considering the average M_r value of 38,000 obtained after SDS-PAGE (Figure 6), it appears unlikely that this DHY-CYC protein is monomeric. Also, failure to detect subunit proteins of differing mass after SDS-PAGE suggests that the protein is probably not a heterodimer. D'Ari and Rabinowitz (1991) have noted that all of the 5,10-CH_2-H_4PteGlu dehydrogenases characterized to date are homodimeric, irrespective of their mono-, di- or tri-functional nature. This generalization is probably valid for this plant dehydrogenase as the derived amino acid sequence, based on the isolated cDNA (Figure 23) had a M_r value of 31,344. This is consistent with the native M_r of this protein determined by gel filtration studies (Figures 7 and 8). In this regard, a similar molecular weight was obtained for the bi-functional DHY-CYC in *E. coli*, where the calculated M_r of the derived amino acid sequence from its encoding gene is 31,060 Da, whereas the subunit size determined by SDS-PAGE is 35 kDa (D'Ari and Rabinowitz, 1991). For the pea protein, the higher value (38 kDa) determined by SDS-PAGE may be erroneous as the protein has a relatively high isoelectric point (8.46). Consequently, this would give the protein a high overall positive charge, which partially counteracts the negative charge resulting from SDS treatment. As a result, the protein would have a lower mobility during SDS-PAGE and thus a higher value of subunit mass.

A separate SYN protein, which lacked DHY and CYC activities, was also isolated from pea leaf extracts (Table 7). The purified SYN protein had a subunit M_r of 66,000 as revealed by SDS-PAGE (Figure 12) and a native M_r, determined by gel filtration chromatography on Sephacryl S-300, of 130,000 (Figure 13). In this regard, the pea SYN protein is similar to that isolated from spinach leaf extracts by Nour and Rabinowitz (1991). Cloning and sequencing of the spinach gene encoding this protein (Nour and Rabinowitz, 1992) indicated homologies with the mono-functional bacterial SYN protein and the SYN domain of the mammalian and yeast tri-functional C_1-THF synthases. These authors concluded that spinach SYN is catalyzed by a mono-functional protein. The present work suggests that a similar protein is expressed in the cytosolic compartment of pea leaves.

The purified cytosolic SYN and DHY-CYC proteins were used to raise polyclonal antibodies in rabbits, respectively. The high titers of the antisera, determined by ELISA tests (Figures 9 and 14), suggest that both polyclonals had a high degree of selectivity and sensitivity (Tijssen, 1985). Prior to being utilized in immunological studies, DHY-CYC-Ab and SYN-Ab were partially purified by the fractionation of antisera to isolate IgGs, which are rich in antibodies (Johnstone and Thorpe, 1987). The antibodies raised and purified by these methods were effective in detecting immunologically related proteins in mitochondrial extracts as discussed in Section 4.3.

4. 3. Mitochondrial SYN, DHY and CYC Activities in Pea Leaves

During the investigations of these mitochondrial enzymes, the same precautions were taken to prevent proteolysis as in the purification of their cytosolic counterparts. Despite these precautions, attempts to purify these mitochondrial activities were not successful. All three activities were present at low levels (Table 5) and each displayed relatively poor stability. These mitochondrial extracts however did contain proteins that strongly cross-react with the DHY-CYC-Ab and SYN-Ab respectively when examined by ELISAs (Figures 17 and 18). Furthermore, Western blots analyses (Figure 10) showed that DHY-CYC-Ab detected a mitochondrial cross-reacting protein of approximately the same subunit size as the purified, cytosolic DHY-CYC. In addition, most of the mitochondrial DHY and CYC activities were retained when extracts were passed through an immunoaffinity column containing bound DHY-CYC-Ab (Table 13). Thus it appears likely that pea leaf mitochondria have DHY and CYC activities that are associated as in the cytosolic protein. This association does not appear to include SYN as the bulk of this mitochondrial activity passed through the immunoaffinity column (Table 13) and SYN-Ab did not cross-react with mitochondrial protein of approximately 38 kDa (Figure 15). Also, during the storage of intact mitochondria or their extracts, the mitochondrial SYN activity displayed different stability characteristics from those of mitochondrial DHY and CYC (Table 12). In these respects, the structural organization of DHY, CYC and SYN in pea leaf mitochondria appear to be distinct from that reported for yeast and mammalian mitochondria where all three activities are associated with a protein of subunit M_r of approximately 110,000 (Shannon and Rabinowitz, 1986; Appling, 1991).

The mono-functional SYN proteins characterized to date exhibit homology with the synthetase domain of C_1-THF synthases and have a subunit mass of approximately 60 kDa (Whitehead and Rabinowitz, 1988; Lovell et al., 1990; Nour and Rabinowitz, 1992). It is therefore surprising in the present study that the SYN-Ab, raised against the purified cytosolic SYN, only cross-reacted with mitochondrial proteins of 40 and 44 kDa (Figure 15). The 44 kDa protein was also detected in the whole-leaf extracts (Figure 15). Failure to detect mitochondrial protein of approximately 60 kDa was not due to insufficient antibody titer as these SYN-Ab preparations readily detected comparable low levels of cytosolic SYN (Figure 15). It was also highly unlikely that these proteins were proteolytic fragments as PMSF was included in all buffers, and fresh samples were prepared immediately before being examined by SDS-PAGE. Plausible explanations for these observations might include the following. First, pea mitochondrial SYN is not structurally related to cytosolic SYN and is therefore not detected in the Western blot analyses. This appears unlikely because of the high degrees of homology shown by SYN proteins of diverse species (Nour and Rabinowitz, 1992). Second, the cross-reacting 40 and 44 kDa proteins may have some structural similarity to SYN but do not have this catalytic activity. In this regard, leaf mitochondria contain significant amounts of T-protein (subunit M_r approximately 41,000) as part of the GDC complex (Bourguignon et al., 1993). T-protein also binds tetrahydrofolate and has some structural similarity to the SYN domains of mammalian C_1-tetrahydrofolate synthases (Kopriva et al., 1995). It should be noted however that the expression of T-protein in pea seedlings is strongly enhanced by light (Bourguignon et al., 1993; Turner et al., 1993) whereas the amount of the 44 kDa, SYN-

Ab cross-reacting protein in pea leaves decreased on exposure to light (Figure 21). A third possibility is that the 40 and 44 kDa proteins are structurally related to cytosolic SYN and represent unique mitochondrial forms of this enzyme. As the SYN activities of yeast and mammalian mitochondria are integral parts of a tri-functional protein, the minimal structural requirements for a mono-functional SYN in plant mitochondria remain to be determined. It is clear that these aspects of mitochondrial folate metabolism require more detailed study.

4.4. The Primary Structure of Pea Cytosolic DHY-CYC Protein and Its Encoding cDNA

In order to isolate the cDNA encoding pea DHY-CYC protein, two λgt11 cDNA expression libraries were screened using DHY-CYC-Ab in combination with radiolabeled probes. One of the two libraries was constructed using 10 d light-grown pea leaves (designated L3), whereas the other library was from 9 d dark-grown pea leaves (designated D3). As the expression of DHY-CYC was found to be highly up-regulated by light (Figures 20 and 22), the L3 library was screened first. This unfortunately did not result in the isolation of a nucleotide sequence that encoded the full length amino acid sequence of DHY-CYC (Figure 23). A cDNA clone, designated D3-1 (Figure 23), was finally obtained by further screening the D3 library using a radiolabeled 377bp restriction fragment derived from an incomplete clone (see Sections 2.16 and 3.6).

The D3-1 insert contains 1219 nucleotide residues (Figure 23). Within the open reading frame, the derived amino acid sequence contains 294 amino acid residues from the first methionine to the downstream stop codon. Northern blot analyses of pea leaf total RNA (Figure 24), probed with radiolabeled insert fragment from clone Dλ2, revealed a single transcript band of 1.2 kb. This value is in close agreement with the size of D3-1 insert, indicating the integrity of this cDNA clone. This cDNA clone most likely encodes the cytosolic form of DHY-CYC based on the following observation. There is a stop codon (TAA) which occurs 42 bases upstream of the start codon ATG for the first methionine (Figure 23). This precludes the existence of a mitochondrial or chloroplast targeting signal because the first methionine corresponds to the sequences of the mature form of the protein in other species (Figure 25). The presence of a methionine at the amino terminus of the derived amino acid sequence (Figure 23) strongly suggests that the pea DHY-CYC protein is a bi-functional complex not an artifact of proteolysis during purification. Unfortunately, attempts to determine the amino acid sequence of the purified DHY-CYC protein were not successful due to an apparent block at the amino terminus. In yeast, the cytosolic C_1-THF synthase is encoded by the *ade-3* gene (Appling and Rabinowitz, 1985b; Staben and Rabinowitz, 1986; Barlow and Appling, 1990), whereas the mitochondrial isoform is encoded by the *MIS 1* gene (Shannon and Rabinowitz, 1986 and 1988; Appling 1991). Nevertheless, the subunits of both isoforms are approximately 110 kDa and are immunologically closely related (Shannon and Rabinowitz, 1986). Conceivably, like yeast, pea may also have a distinct gene, differing from the one represented in the D3-1 clone, that encodes the mitochondrial form of DHY-CYC protein. More studies of the enzymology and molecular biology of this mitochondrial protein are needed to resolve this interesting question.

Joshi (1987) compared the start sites of 79 plant genes and proposed the consensus sequences of TAAACAATGGCT (on the plus strand of DNA) for the initiation of translation. The corresponding sequence around the start codon of D3-1 insert for pea DHY-CYC is GATCAAATGGCC, with 7 out of 12 bases identical to the proposed consensus initiation sequence. Codon usage in the D3-1 insert was examined, and it was determined that 37% of codons end in either G or C. This is consistent with the observation that the nuclear genes of dicotyledonous plants have a preference for codons ending in an A or U (Campbell and Gowri, 1990).

Two major protein databases (EMBL-24 and GenBank 65) were searched for the primary structures of the bi-functional DHY-CYC proteins and the C_1-THF synthases of other biological sources. Seven such sequences were obtained namely, human (Hum et al., 1988) and rat (Thigpen et al., 1990) tri-functional C_1-THF synthases, yeast mitochondrial (Shannon and Rabinowitz, 1988) and cytosolic (Staben and Rabinowitz, 1986) tri-functional C_1-THF synthases, the *E. coli* bi-functional enzyme (D'Ari and Rabinowitz, 1991), and the human (Peri et al., 1989) and mouse (Belanger and MacKenzie, 1989) bi-functional mitochondrial enzymes. These sequences were aligned with the pea bi-functional protein as shown in Figure 25. For the tri-functional enzymes, only the DHY and CYC domains were used. The alignment shown is a composite of the PC/GENE Clustal program with further refinement done visually. Figure 25 shows that there are several large blocks of homology common to all eight sequences. D'Ari and Rabinowitz (1991) compared the amino acid sequence of *E. coli* bi-functional DHY-CYC with the other six sequences listed above. They found a 14-amino acid sequence namely, ITPVPGGVGPMTVA, which is present in all seven proteins with no substitutions or gaps. This sequence was not found in any other protein contained in the databases examined by D'Ari and Rabinowitz (1991). The alignment shown in Figure 25 reveals that the pea bi-functional DHY-CYC protein also contained this consensus sequence (residues 263-276) except for the last amino acid A, which was replaced by T. The uniqueness of this amino acid plus three other residues in pea (Ala-146, Ala-214, and Arg-246), which were not found in the conserved amino acid residues shared by the other seven species, indicates that the higher plant enzymes may have catalytic characteristics that are slightly different from those of other species.

The degree of identity of pea DHY-CYC sequence, relative to each of the seven sequences mentioned above, was examined by the Palign program of PC/GENE (Table 15). The yeast mono-functional NAD-dependent dehydrogenase (West et al., 1993) was also included in this comparison. The percentage identity of these individual comparisons (Table 15) were very similar, except for the yeast mono-functional dehydrogenase. This was a surprise because the DHY of the two bi-functional mitochondrial enzymes are specific for NAD, while the DHY of pea and *E. coli* bi-functional enzymes, as well as the four tri-functional C_1-THF synthases were NADP-dependent. The highest percentage identity was obtained when the pea protein was compared with the yeast cytosolic C_1-THF synthase. This is consistent with the observation that DHY-CYC-Ab strongly cross-reacted with this yeast protein (Table 11). As expected, the lowest value of 25.2% occurred when the pea bi-functional protein was compared with the yeast mono-functional dehydrogenase. Since the DHY and CYC activities of the C_1-THF synthase are kinetically dependent (Drummond et al., 1983), and appear to share a common folate-binding site (Smith and MacKenzie, 1985), it follows that the yeast mono-functional dehydrogenase probably lacks most amino acid residues that are required for the CYC activity, even though its primary structure (320 amino acids) is similar in size to the bi-functional enzymes and domains.

4.5. SYN, DHY and CYC Activities in Relation to Photorespiration

The data in Figure 20 show that greening of etiolated pea seedlings was accompanied by increased levels of DHY and CYC activity in whole-leaf extracts. This treatment did not appear to affect the specific activities of DHY or CYC in mitochondrial extracts (Table 14). These greening conditions resulted in a progressive loss of SYN activity in whole-leaf and mitochondrial extracts (Figure 20; Table 14). Western blot analyses (Figure 21 and 22) showed that greening had similar effects on the levels of the cross-reacting proteins. It follows that greening was accompanied by an enhancement in the synthesis or turnover of these folate-dependent enzymes. During the development of photorespiration in greening tissues, the expression of two other folate-dependent enzymes, GDC and SHMT, is strongly up-regulated (Turner et al., 1993; Vauclare et al., 1996; Guinel and Ireland, 1996). As GDC catalyzes the mitochondrial formation of 5,10-CH_2-$H_4PteGlu_n$, it follows that greening will provide substrate for mitochondrial DHY and SHMT. It is likely that most of this folate substrate will be utilized in serine biosynthesis as the specific activity of SHMT is about 15-fold higher than that of DHY in pea leaf mitochondria (Neuburger et al., 1996). Conceivably, the metabolic role of mitochondrial

DHY and CYC is the conversion of 5,10-CH_2-H_4PteGlu$_n$ to 10-HCO-H_4PteGlu$_n$, which is needed for the synthesis of formylmethionyl-tRNA, the initiator of protein synthesis in these organelles (Staben and Rabinowitz, 1984; Coffin and Cossins, 1986). A related study has recently shown that 10-HCO-H_4PteGlu$_n$ is a component of the mitochondrial folate pool of pea leaves (Chen et al., 1997). The relatively low levels of mitochondrial SYN activity (Table 14) after greening suggest that the activation of formate is probably a minor route for 10-HCO-H_4PteGlu$_n$ biosynthesis in mitochondria of photosynthetic tissues. Thus it is concluded that pea mitochondria probably generate this folate by DHY and CYC activities during photorespiration.

During photorespiration, serine is exported by mitochondria and converted to glycerate by peroxisomal enzymes (Douce and Neuburger, 1989). As the cytosolic compartment of leaves contains SHMT (Somerville and Ogren, 1981; Neuburger et al., 1996) and an *Arabidopsis* mutant, deficient in mitochondrial SHMT, grew normally under non-photorespiratory conditions (Somerville and Ogren, 1981), it follows that some of this exported serine may be available for the biosynthesis of 5,10-CH_2-H_4PteGlu$_n$ by the cytosolic SHMT. In other eukaryotes, cytosolic pools of this folate have importance in the formation of thymidylate, purines, methionine and choline (Schirch, 1984). In mammalian cells, the utilization of 5,10-CH_2-H_4PteGlu$_n$ in purine biosynthesis is facilitated by a functional interaction between cytosolic SHMT and C_1-THF synthase (Strong and Schirch, 1989). There is still relatively little information on the biosynthesis of purines in plants (for review see Cossins and Chen, 1997), but it is conceivable that green tissues generate the 10-HCO-H_4PteGlu$_n$ needed in this pathway by cytosolic DHY-CYC activity.

Thus during photorespiration some of the serine formed in the mitochondria may enter a cytosolic pathway that generates C-1 substituted folates for other pathways of one-carbon metabolism.

4.6. Structural Organization of SYN, DHY and CYC in Other Higher Plant Species

The present study also examined other higher plant species for the occurrence of SYN, DHY and CYC activities. These included the angiosperms: bean (*Phaselous vulgaris*), barley (*Hordeum vulgare*), oats (*Avena sativa*), corn (*Zea mays*), wheat (*Triticum aestivum*); and the gymnosperms: pine (*Pinus taeda*) and ginkgo (*Ginkgo biloba*). This survey also included two fungal species: *Neurospora crassa* and *Agaricus brunnescens*; a protist, *Euglena* and a bacterium, *Lactobacillus casei*. In most of these species, all three activities were readily detected (Tables 8, 10 and 11) when the standard enzyme assay procedures were followed. Failure to detect DHY activity in *Agaricus brunnescens* and CYC activity in bean, ginkgo and *Euglena* extracts may be due to their low activity levels and the interference of pigments present in the crude extracts. These enzyme proteins may be expressed in these species as SYN-Ab and DHY-CYC-Ab detected cross-reacting protein bands when tissue extracts were subjected to immunoblot analyses (Tables 10 and 11).

During the purification of the cytosolic DHY and CYC from pea leaf extracts, SYN activity was clearly separated from DHY and CYC after Matrex Green column chromatography (Figure 5). The same protocol (Table 6) was therefore used to examine extracts of several other higher plant species. The data shown in Table 8 indicate that in bean, wheat, corn and barley plants, SYN protein is not associated with that of DHY and CYC, as these latter activities were co-eluted from the affinity column and the recovery of each in column elutions (Table 8) was comparable. This organization of the three activities in these plants is also supported by the results of Western blot analyses using SYN-Ab (Table 10) and DHY-CYC-Ab (Table 11). Thus in all surveyed higher plant species, the apparent M_r values of the DHY-CYC-Ab cross-reacting protein bands were distinct from those of proteins which cross-reacted with SYN-Ab. The strong cross-reactivity shown in ELISA (Table 9) and immunoblot analyses (Tables 10 and 11), also indicate that DHY, CYC and SYN proteins in these species are structurally related to their counterparts in pea.

These immunological studies are in many respects similar to the earlier work of Staben and Rabinowitz (1983). These workers showed that antisera to yeast cytosolic C_1-THF synthase cross-reacted with the corresponding synthase protein of several other eukaryotic species and with the mono-functional 10-HCO-H_4PteGlu synthetase protein of *Clostridium acidi-urici*. They also reported that antibodies raised against the purified bacterial synthetase cross-reacted with the C_1-THF synthase proteins of several eukaryotic species. Immunoblot analyses confirmed the specificity of this cross-reaction and the authors concluded that all of these enzymes have common structural features. Furthermore, Nour and Rabinowitz (1992) have shown that the primary structure of spinach SYN contains most of the conserved sequences found in SYN proteins or SYN domains of other sources. In the present work (Figure 25; Table 14), it is clear that pea

DHY-CYC protein also shares most of the conserved amino acid sequences with the corresponding proteins of other species. Based on the present data (Tables 8, 9 10 and 11), it appears that SYN and DHY-CYC proteins of similar structure to those of pea leaves also occur in other plant species. In *Neurospora*, the presence of cross-reacting protein bands of different M_r, when probed with SYN-Ab (Table 10) and DHY-CYC-Ab (Table 11) respectively, raises the possibility that not all of the SYN, DHY and CYC activities of this fungus are associated in a C_1-THF synthase. Conceivably, this organism, like yeast, may express an NAD-dependent DHY activity but also a mono-functional SYN. The examination of *Neurospora* mutants, such as the formate mutant, which lacks cytosolic SHMT and over-expresses SYN (Cossins, 1987) could provide basic information in these areas of folate-dependent one-carbon metabolism.

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